

Author: David E. Mahony

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A TEMPERATE BACTERIOPHAGE OF  
CLOSTRIDIUM PERFRINGENS

by

DAVID E. MAHONY, B.Sc. (Acad.), M.Sc. (Dal.)

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Department of Microbiology and Immunology  
McGill University  
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David E. Mahony

A TEMPERATE BACTERIOPHAGE OF CLOSTRIDIUM PERFRINGENS

A temperate bacteriophage was isolated from a lysogenic strain of Clostridium perfringens. This strain was inducible by ultraviolet light and differed from the phage indicator strain by the absence of a haemolysin and pathogenicity for guinea pigs. Preliminary experiments did not reveal a phage-haemolysin relationship.

One-step growth curves conducted in broth containing a reducing agent revealed latent and release periods of 45 minutes each. Phage yields were variable but, in general, quite low. Plaques produced on solid medium were small and possessed turbid centers. Lysogenization of the indicator strain occurred.

The phage had a polyhedral head 80 x 70 m $\mu$  and a striated tail 200 m $\mu$  long as determined by electron microscopy. Experiments involving temperature, ultraviolet light and pH stability of the phage as well as adsorption experiments were performed.

The phage nucleic acid, as determined by the diphenylamine reaction, acridine orange staining and sensitivity of phage multiplication to mitomycin C, was deoxyribonucleic acid.

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## INTRODUCTION

The concept of lysogeny and temperate bacteriophage has been of interest throughout the history of bacteriophage investigations. How the phage nucleic acid becomes incorporated into the bacterial genome and why it remains in a latent state only to be prompted to direct the synthesis of new phage particles upon various stimuli, some of which are known and others not, have been two major and controversial questions pertaining to the lysogenic state.

Another very interesting aspect of lysogeny was revealed by Freeman in 1951 when he demonstrated that a temperate phage derived from a lysogenic and toxigenic strain of Corynebacterium diphtheriae could convert a non-toxigenic strain to a toxigenic strain. This phenomenon was shown to be a property governed by the phage genetic material, and not due to the transduction of bacterial genes by a phage, since every phage was capable of "converting" the indicator strain. Interest turned to other toxin systems where lysogeny was known to exist. In 1964 Zabriskie established that a temperate bacteriophage was responsible for controlling the synthesis of erythrogenic toxin in Group A streptococci and in 1965 Winkler, deWaart and Grootzen showed that the presence of a temperate bacteriophage could repress Beta-toxin

formation in staphylococci. Other examples of "phage conversion" exist, but they do not involve toxin production.

Although a great number of phage isolations have been made throughout the years, it was noted that very little work had been conducted on the bacteriophages of the genus Clostridium. Such studies might be worthy in view of the number of toxins produced by various members of this genus, in particular those of Cl. perfringens. Perhaps such toxins are influenced by bacteriophage as in the above mentioned examples of phage conversion. A few virulent phages have been described for Cl. perfringens, and a paper by Smith (1959) revealed the existence of lysogenic strains of this species, but a temperate phage has never been characterized.

The aim of this investigation was, therefore, to isolate and characterize a temperate bacteriophage of Cl. perfringens and, if possible, determine whether any phage-toxin relationships might exist.

## HISTORICAL

### Lysogeny and Temperate Bacteriophage

#### Introduction and Review

In many instances phage-bacterial host relationships involve the infection of a susceptible bacterial host cell by a particular phage, multiplication of this phage within the host cell and then, as a final step, the bursting or lysing of the bacterial cell accompanied by the release of a number of new phage particles. These released phage particles can then go on to infect other susceptible bacterial cells and the cycle is repeated. Such a cycle is referred to as the lytic response and phages which elicit such a response are called virulent phages. However, there is another very important aspect of phage-host relationships which is called lysogeny, a state in which phage can be perpetuated as part of the bacterial hereditary apparatus during normal growth of the host cell. Phages eliciting this response can be classed as temperate phages, these being able to produce either a lytic response, or, in contrast, the lysogenic state.

Every lysogenic bacterium carries a complement of phage genes in the form of a prophage. Such a relationship renders the bacterium immune from attack by homologous phage,

with the deoxyribonucleic acid (DNA) of the superinfecting phage being unable to engage in the vegetative replication known to occur in non-lysogenic cells. In a lysogenic population a cell will lyse from time to time with the release of a number of bacteriophage particles. When such a population is exposed to various physical or chemical agents, a great percentage of the cells may lyse with the production of bacteriophage. This process is called induction. Thus, the lysogenic state confers upon the bacterial cell the ability to liberate bacteriophage, immunity to attack by homologous phage and an increased sensitivity to inducing agents (Jacob and Wollman, 1953; 1956).

It is of interest at this point to briefly review how the concept of lysogeny and temperate bacteriophages developed. Following the independent discoveries by Twort and d'Herelle in 1915 and 1917 concerning lytic factors in bacteria, many workers became interested in the bacteriophage phenomenon. Twort was of the opinion that bacteriophages had their origin in the bacterial cell, whereas d'Herelle proclaimed that bacterial strains possessing these lytic factors were strains contaminated with phage and could be made rid of their phage by purification methods. Bordet, in 1925, obtained one of the first pictures of lysogeny by showing that all the bacteria of a lysogenic strain produced clones which were lysogenic, this property being maintained in the absence of

free phage. McKinley (1925) further verified this observation by showing that antiserum against phage had no effect on destroying the lysogenic state. Bordet and Renaux (1928) showed that each descendant of every lysogenic bacterium produced phage. In 1929 Burnet and McKie showed that, by inducing lysis of a lysogenic strain of Salmonella enteritidis with an extrinsic phage unrelated to the carried phage, only 0.1 per cent of the lysogenic bacteria liberated any intracellular phage. Since no infective virus was shown to be present, it was proposed that the lysogenic bacteria perpetuated the power to produce infectious phage as a non-infectious "anlage", which multiplied in step with the cell. Infectious phage would only be produced when the "anlage" was activated. Wollman and Wollman (1936, 1937) independently came to the same conclusion, calling the factor involved "forme intracellulaire du phage". Den Dooren de Jong (1931), Cowles (1934) and Northrop (1951) also suggested that a different form of phage existed by showing that the heating of spores of a lysogenic culture did not destroy their ability to produce phage upon germination. Such treatments would normally destroy the infectivity of bacteriophage.

Lwoff and Gütman (1950) demonstrated that, by micro-manipulation of single cells, lysogeny persisted for at least 19 successive generations in the absence of free phage. Lysis was always followed by a release of phage and each cell yielded

a burst of many phage particles (also Clarke and Cowles, 1952). The name given to the latent lytic factor was prophage. Thus it was concluded that each bacterium of a lysogenic strain harbours and maintains a non-infectious structure, the prophage, which endows the cell with the ability to give rise to infective phage without the intervention of exogenous phage particles (Lwoff, 1953).

Lysogenization, then, refers to the action of conferring to a bacterium the hereditary power to produce a given phage. Bail and Bordet (1925) had discovered that when non-lysogenic bacteria were mixed with certain phages, some of the surviving clones were lysogenic. Lwoff (1953) suggested that the lysogenic response of a bacterium was controlled by the genetic constitution of the bacterium and of the phage, by the interaction of these genetic structures, by the phenotypical traits of the bacterium, by its metabolism and perhaps by the multiplicity of infection.

#### Factors Influencing Lysogenization

The capacity to lysogenize does appear to depend upon a number of factors. Fry and Gros (1958) showed that with  $\lambda 22$  phage giving a lysogenic response in Escherichia coli K 12 there was a complete block of protein, DNA and RNA (ribonucleic acid) synthesis which lasted for 60 - 100 minutes. With temperate phages giving a lytic response the synthesis of proteins and nucleic acids



was noted to continue. These authors suggest that the cessation of metabolic processes form an environment in which the genetic material of the phage can attach itself to the bacterial chromosome and thus bring about the establishment of prophage. This pattern for  $\lambda$  phage may be an exception to the rule according to Whitfield (1962). Exposure of cells to chloramphenicol during the early stages of phage infection was shown to increase the frequency of lysogenization in E. coli, S. typhimurium and Shigella dysenteriae with the temperate phages P2, P22 and P1, respectively (Bertani, 1957; Christensen, 1957). Treatment of the cells with amino acid analogues, 5-hydroxyuridine or starvation for a required amino acid also increased the frequency of lysogenization.

Temperature is another factor influencing the efficiency of lysogenization. Various workers have reported that temperatures in the vicinity of 20° C favoured the lysogenic response, while higher temperatures favoured the lytic response (Bertani, 1953; Lieb, 1953; Jacob and Wollman, 1959; Glynn and Baily, 1961). Levine and Hamilton (1964) have shown that there is a close correlation between temperature sensitivity and DNA inhibition with phage P22.

A few workers have suggested that the stage of cell division may play a role in the determination of the lysogenic response (Lark et al., 1955) although other workers refute this

suggestion (Boyd and Bidwell, 1964) with rather convincing evidence.

The multiplicity of infection (m.o.i.) has also been associated with the degree of lysogenization. Groman (1953) noted a higher lysogenic response with higher phage to bacteria ratios in Corynebacterium diphtheriae within a short time interval.

Others have reported the complete antithesis for the same organism (Barksdale and Pappenheimer, 1954). Lieb (1953), referring to

$\lambda$  phage and E. coli, suggested that one phage per bacterium was adequate for lysogeny to occur, but a higher m.o.i. gave a greater number of lysogenic cells. Jacob and Wollman (1959) noted that, with S. typhimurium infected with phage A, a m.o.i. of less than one gave a mostly virulent response. As the m.o.i. increased up to ten phages per bacterium, lysogeny approached 100 per cent.

Boyd and Bidwell (1961) studied this organism also and thought that there were two phage types present,  $\alpha$  and  $\beta$ ;  $\alpha$  produces lysogeny and  $\beta$  produces lysis. However, with higher m.o.i.'s which  $\alpha$  phage could not explain, more lysogeny occurred. It was conceived that two  $\beta$  phages could enter a cell and exert a lysogenic response by their combined presence. Kaiser (1957) demonstrated that mutants of  $\lambda$  phage which gave clear lysis with little or no lysogeny, when in certain pairs, together produced a high frequency of lysogenization characteristic of the wild

type. Levine (1957) reported a similar phenomenon in S. typhimurium. This information suggests the important role of the genetic constitution of a phage in the development of lysogeny.

These factors, then, represent some of the influential forces which have been observed to play a role in the process of lysogenization. Undoubtedly many others exist.

#### The Nature of Prophage

The nature of the prophage is not too well understood although it has been the subject of many investigations. Prophage appears to evolve, upon lysogenization, from the DNA of the infective phage particle. Some workers believe that there is an initial replication of phage before lysogeny occurs (Stent and Fuerst, 1956; Jacob, Fuerst and Wollman, 1957; Whitfield, 1962; Uetake et al., 1963), while others do not believe that free multiplication is possible and that the phage genetic material is taken up into the host nuclear material in a very short time (Boyd and Bidwell, 1964). The incorporation of the phage genome may take place by genetic recombination or by copy choice with no material change between parental strains and their recombinants. Assuming that the number of prophage units per bacterium is small, it is implied that both the replication of prophage and its distribution to daughter bacteria must in some way be tied to the replication

and distribution of the bacterial genome. The prophage nucleic acid may be so closely related to the bacterial chromosome that it behaves in all relevant respects like a bacterial gene. There appears to be no more than one prophage of the same type per bacterial nucleus (Jacob and Wollman, 1953).

Although many theories exist to explain the mode of attachment of the phage genome to the host chromosome, no one theory suffices. Two theories have become more popular and these are briefly presented here (Hayes, 1965). The first theory describes the attachment of the C region of the prophage chromosome to a specific area of the host chromosome. Since this is a small area of attachment, it is suggested that the rest of the phage genome would drag along from this point of attachment. The second theory implies that there is an insertion of the phage genome into the bacterial chromosome either as an addition to it or a replacement for some bacterial component. This latter model, originally suggested by Campbell in 1962, has become more widely accepted in recent years for explaining various genetic phenomena.

It was found that the DNA of  $\lambda$  phage (one of the better studied temperate bacteriophages and active on E. coli) and, indeed, that of other related temperate phages, was converted from a linear to a circular molecule within the bacterial cell by covalent bonding between complementary bases at the terminal ends of its double

strands (Dove and Weigle, 1965; Bode and Kaiser, 1965; Edgar 1965; Baldwin et al., 1966; Gellert, 1967; Salzman and Weissbach, 1967), and that this circular molecule became inserted into the host chromosome and, hence, part of it (Calef and Licciardello, 1960; Rothman, 1965; Baldwin et al., 1966).

Once a prophage is established, a state of immunity develops in which the prophage multiplies in synchrony with the bacterial chromosome of which it is now a part. Vegetative multiplication of superinfecting homologous phages is prevented and no phage functions involved in maturation or lysis are expressed (Luria, 1962). Prophage induction in a lysogenic population under normal conditions is relatively rare and only a small fraction of the bacteria releases phage particles (Bertani, 1951; Six, 1959). The ratio "free phage/bacteria" remains constant during the exponential phase of bacterial growth. Since the individual burst sizes conform to a normal distribution, there is a constant probability of phage production per physical unit of time (Six, 1959). The actual rate would depend on the host type and location of the prophage on the bacterial chromosome. Theories which attempt to explain the suppression of prophage and immunity will be discussed further on.

### Induction of Prophage

A considerable range of agents, both physical and chemical, has been utilized to bring about induction of prophage. The first demonstration of induction was performed by Lwoff, Siminovitch and Kjeldgaard in 1950 when these workers successfully induced lysogenic Bacillus megaterium with ultraviolet (u.v.) light. They found that complete lysis occurred when the bacterial cells were irradiated in fluid medium with a u.v. lamp emitting a wavelength of  $2537 \text{ \AA}$ . A lag period, which increased with higher doses of irradiation, was noted between irradiation and phage release. Other lysogenic bacteria were found to be inducible by u.v. light such as E. coli K 12 ( $\lambda$ ) (Weigle and Delbrück, 1951), staphylococci (Cavallo and Cantelmo, 1951) and Pseudomonas pyocyanea (Jacob, 1950, 1952a). All workers observed that the optical density of a growing culture continued to increase after ultraviolet treatment, although at a slower rate than the control culture, and then lysis occurred. The latent period for induced phage was greater than that of a sensitive strain infected with virulent phage by twenty to thirty minutes. Glucose and essential amino acid starvation of the bacterial cells was shown to influence the efficiency of u.v. irradiation in induction experiments (Jacob, 1952b; Borek, 1952), and the effect of various ions on induction was also observed (Lwoff, 1952; Huybers, 1953).

Cytological observations made on lysogenic B. megaterium (Delaporte and Siminovitch, 1952) revealed that after u.v. irradiation the cells remained normal for a period, followed by elongation and nuclear and cytoplasmic increase. Weigle and Delbrück (1951), observing induced E. coli K 12, showed that the bacteria double in length and thickness during the latent period but do not divide. At the end of the latent period they assumed a spherical shape and burst a few seconds later, leaving a mass of debris.

Other physical methods of induction have also been found. Shortly after the discovery of u.v. induction, Latarjet (1951) reported a similar response using x-rays and lysogenic B. megaterium. The lag period was shorter than that obtained with u.v. induction, but the per cent induction was found to be less (Tobin, 1953). The control of inducing conditions was found to be similar to that required for successful u.v. induction. Marcovich (1956) studied x-irradiation on E. coli K 12 and found that the age of the culture had little effect on x-ray induction. Stronger doses of x-rays did not increase the latent period, but rather decreased the number of surviving cells.

A heat shock has been shown to induce some prophages (Cavallo, 1951; Soller and Epstein, 1965; Sugino, et al., 1966) and Lieb has recently reported detailed genetic studies on heat-inducible lambda bacteriophage (1966a, b, c; 1967). A group of

Russian workers (Solovieva et al., 1965) have demonstrated that mycelia and spores of Actinomyces fradiae can be induced to form phage by means of ultrasonic sound. Another experimental technique showed that a hydrostatic pressure of 720 atmospheres could induce E. coli K 12 ( $\lambda$ ) (Rutberg, 1965).

A number of chemical substances can also induce lysogenic systems. Lwoff and Siminovitch (1952) demonstrated that several reducing chemicals could induce lysogenic B. megaterium, thiomalic and thioglycollic acids being the most efficient and easiest to work with. Hydrogen peroxide, many epoxides, ethyleneimines and halogenoalcoylamines were also effective inducers in certain lysogenic bacteria (Lwoff and Jacob, 1952) as well as was bis-(B-chloroethyl)-methylamine, which was mutagenic and carcinogenic (Jacob, 1952c). Smith studied the effect of a number of substances as inducers on lysogenic strains of Salmonella (1953) and Clostridium perfringens (1959).

An antibiotic, phagolessin A 58, which inactivates bacteriophages (Hall and Asheshov, 1953), served to induce a number of lysogenic strains (Hall-Asheshov and Asheshov, 1956). Another inducing agent was found by Japanese workers (Otsuji et al., 1959) in the drug mitomycin C, which, in E. coli K 12, produced a 200-fold increase in phage production after addition to the culture. DNA synthesis was prevented selectively. Mitomycin C has also



been found to induce such organisms as Xanthomonas campestris (Sutton and Quadling, 1963) and Bacillus stearothermophilus (Welker and Campbell, 1965 a, b). In comparing the effect of u.v. with mitomycin C in the latter organism, it was found that u.v. repressed the RNA synthesis for 15 minutes after which the synthesis returned to normal, whereas mitomycin C had no effect on RNA synthesis. Both agents repressed DNA synthesis for the first stage of the latent period after which synthesis increased to three times that of the control. Protein synthesis was slightly inhibited by these agents for a short period.

The deprivation of a certain metabolite may cause induction of prophage. Such is the case in an auxotrophic mutant of E. coli B-3, which requires thymine for growth. If such bacteria are lysogenic, thymine starvation for 90 minutes followed by the addition of thymine again results in maximum induction of the strain (Melechen and Skaar, 1960; Maisch and Wachsman, 1964). Presumably through the interruption of DNA synthesis thymine deprivation leads to induction of these lysogenic bacteria.

Like mitomycin C, L-azaserine is an antineoplastic agent and it has been found to induce lysogenic E. coli K 12 (Gots et al., 1955). Price, Buck and Lein (1964) have drawn a relationship between carcinogens, antineoplastic agents and inducers of prophage, using E. coli as the experimental test organism. Carcinogens do

not induce lysogenic bacteria in most cases; only a few do, and these are water soluble. The only generalization which can be made is that all inducers have antineoplastic activity, but many antitumor agents do not cause induction.

The dye acridine orange, in combination with u.v. irradiation, has caused induction in E. coli (Smarda et al., 1965). An explanation for such a phenomenon has been suggested by Lieb (1964) who noted that the addition of proflavine (an acridine) and caffeine (a methyl purine) to the irradiation medium greatly increased u.v. induction. She theorizes that these compounds, which are not inducers in themselves, prevent "dark repair" of u.v. damage, the repair which is increased by photoreactivation.

Other inducing agents have been found, such as B-propiolactone and dimethyl sulphate (Field and Naylor, 1962) and in 1965 Dudnik showed that antibiotics affecting DNA synthesis could induce lysogenic bacteria, whereas those inhibiting protein and RNA synthesis had no effect.

Undoubtedly there are other inducing agents which have been used, but the list presented here suggests that there is a considerable number of agents capable of inducing prophage to multiply vegetatively and that one particular agent or set of conditions will not necessarily work in every strain of lysogenic bacteria. The factors involved in induction would appear to be rather complex.

### Current Theories of Immunity

There is yet one very important type of induction to be mentioned and such an induction has shed considerable light on a mechanism which may possibly explain under which circumstances the prophage is suppressed or can multiply, and why the lysogenic host cell is immune to homologous phage infection. In 1954 Jacob and Wollman noted that during crosses between E. coli K 12 Hfr lysogenic and F- non-lysogenic bacteria, the prophage passed from one parent to the other via the donor chromosome and was induced in the recipient non-lysogenic cell. The resulting zygote was destroyed and the process was called zygotic induction. The theory is held that in lysogenic bacteria the presence of a cytoplasmic repressor prevents multiplication of both the prophage and the superinfecting homologous phage (Jacob and Campbell, 1959). This repressor would not be present in the recipient, non-lysogenic bacterium and when the donor chromosome carrying a prophage enters the cell, the repressor would be absent or markedly diluted out. The level of repressor would be too low to prevent vegetative multiplication. A similar phenomenon was noted when an inducible prophage was transferred from a lysogenic bacterium to a non-lysogenic bacterium by means of transduction (Jacob, 1955).

The repressor theory is an extension of Jacob and Monod's theory concerning the genetic control of enzyme function. This

theory, as it applies to induction of prophage, can be briefly summarized as follows. A cytoplasmic repressor substance synthesized under the control of a regulator gene combines with an operator gene. This association causes the operator gene to inactivate the functioning of the structural genes under its control, which, in this case, are responsible for phage synthesis. There may be a number of regulator loci present governing a great number of operators respectively. A repression or mutation at the regulator level, a physical destruction of the repressor substance, or a mutation at the operator level may account for derepression of a repressed system, i.e. induction.

There is considerable evidence that such a theory may apply to immunity and active phage multiplication in the lysogenic system. The C1 cistron of the  $\lambda$  chromosome appears to be responsible for the synthesis of the repressor, since the prophage is lost when there is a mutation in this region, but not when the mutation occurs in the C2 and C3 regions (Kaiser, 1957; Jacob, 1960; Sussman and Jacob, 1962; Levine and Hamilton, 1964). The actual nature of the repressor has been studied in a number of ways. Studies with F + male bacteria and F - female bacteria, where only cytoplasmic transfer occurs rather than chromosomal exchange, revealed that when F + immune cells were crossed with F - non-immune cells the F - cells became progressively immune to  $\lambda$  phage with increasing

transfer of cytoplasm (Hayes, 1965). This immunity decayed spontaneously with time, but it was immediately destroyed by u.v. irradiation. A similar experiment was performed using a non-inducible lysogenic F + donor strain. Again immunity was transferred passively to the F - cell, but in this case the immunity was not destroyed by u.v. light. This evidence more or less substantiates the presence of a cytoplasmic repressor. The non-inducible systems produce somewhat different types of repressors which are not sensitive to u.v. irradiation.

Wiesmeyer (1966) demonstrated that the concentration of  $\lambda$  repressor molecules could be estimated from the multiplicity of superinfecting homologous phage necessary to take up the repressor substance and release the prophage from repression. The theory is put forward that the superinfecting phage, after entering the lysogenic cell, will combine with the repressor molecules and, if enough phage is added, all the repressor will be used to repress these phages and the prophage will be free to multiply vegetatively.

A very interesting piece of work has recently appeared (Ptashne, 1967a, b) in which the  $\lambda$  repressor has been isolated from lysogenic E. coli and characterized as a protein with a molecular weight of 30,000. This protein has been shown to bind specifically to  $\lambda$  DNA suggesting that the repressor blocks transcription from DNA to RNA.

Although the repressor theory does provide many seemingly logical answers to the problem of induction and immunity, it still may not be the correct or whole answer. There are some lysogenic systems in which the prophage is not inducible by any means. Whether a prophage is of the inducible type or not seems to be a function of the phage, rather than the host bacterium, since the same bacterial strain may be inducible when lysogenized by one type of phage, and non-inducible when lysogenized by another. An inducible phage which is able to lysogenize a number of different hosts is inducible in all of them (Ionesco, 1951; Hayes, 1965). Exceptions to the rule occasionally occur in which the host would seem to be the controlling factor (Uetake, 1959). Perhaps Lwoff (1953) is correct in saying that the mode of equilibrium between host and phage seems to be a factor. A study of 14 different bacteriophages isolated from E. coli, half of which were not inducible, revealed that the inducible prophages were grouped within a segment of chromosome amounting to about one-quarter of its length, while those prophages of the non-inducible type were distributed over the remainder of the chromosome. The significance of this observation is not yet known (Hayes, 1965).

Another phenomenon observed was that certain bacterial strains exhibiting an immunity pattern resembling lysogenic strains, when irradiated with u.v. light, lysed with no release of infective

phage particles (Lwoff and Siminovitch, 1951; Quersin, 1951).

This "abortive development" appeared to be due to incomplete phage development. It was found that the abortive development system produced little DNA during the second half of the latent period, whereas a normal control system rapidly synthesized DNA during this period. Jacob and Wollman (1956) described a similar situation of this defective lysogeny and in the lysates of u.v.-induced E. coli K 12 ( $\lambda$ ) found empty phage heads as well as non-adsorbable, non-sedimentable serum blocking antigens. These workers attributed the prevention of phage maturation to a lesion in the prophage rather than in the bacterium. Induction of the defective strain suggested that the lesion is not in the bacterium (Jacob and Wollman, 1959).

A number of observations have been made on the effect of inducing agents and the response of the prophage leading to conclusions varying from the repressor theory. It was proposed by some workers (Whitfield and Appleyard, 1957) that in the process of induction the prophage is not itself physically converted to a vegetative phage, but makes at least one vegetative replica which initiates vegetative multiplication. It is proposed that the DNA of the host is inhibited by u.v. irradiation, but the DNA of the phage is not. Multiplication of the phage could then occur as an autonomous unit in this unbalanced condition. The host

DNA, itself, may be the repressor (Levine, 1961; Whitfield, 1962). However, it has been shown that, by starvation, mere stoppage of DNA synthesis is not sufficient for induction (Melechen and Skaar, 1962). It has been suggested that during thymine starvation, only a first phase of induction occurs which predisposes the phage DNA to vegetative replication. Protein synthesis accompanied the inhibition of DNA synthesis. These workers hypothesized that induction acted by uncoupling a protein bond between phage and bacterial DNA.

In a later experiment Melechen (1964) found that protein synthesis was not necessary for induction as shown by treatment of E. coli cultures with protein inhibitors. Since all the procedures that result in activation of the prophage probably alter the DNA formed, it was proposed that the synthesis of an abnormal DNA may cause induction of the Plb phage studied. Experiments with 6-azauracil (Zgaga and Miletic, 1965) indicated that the first step in the process of induction, which causes loss of immunity, was reversible and did not automatically lead to induction of the prophage.

In summary, two major theories involving immunity in lysogenic bacteria and induction of prophage have been discussed - one emphasizing a repressor mechanism, the other suggesting an alteration in host DNA or shift in the balance of a delicately



poised system. Recent studies seem to have shed more light on the repressor model of immunity and more information on this subject will have to come from the field of molecular genetics.

#### Phage-host Relationships

The relationship of the prophage and/or the bacteriophage to the host bacterium in toxin producing Corynebacterium diphtheriae (Freeman, 1951; Barksdale, 1959; Miller, Pappenheimer and Doolittle, 1966), streptococci (Zabriskie, 1964) and staphylococci (Blair and Carr, 1961; Winkler, de Waart and Grooten, 1965), as well as in altering staphylococcal phage typing patterns (Blair and Carr, 1961), extracellular lipase production (Rosendal, 1964) and the antigenic structure of various Salmonellae (Uetake et al., 1955; Zinder, 1957; Stocker, 1958; Uetake et al., 1958; Barksdale, 1959) serves to emphasize the importance of studying lysogeny, the prophage and various interactions of bacteriophage and the host. In light of the fact that spontaneous induction of lysogenic cells from time to time may be responsible for toxin production in some systems, that such a response is a major curiosity in itself, and that induction by various agents can greatly accelerate normally random occurring events in Nature, the topic of lysogeny and temperate bacteriophages becomes of worthy interest.

## Clostridial Bacteriophages

### Introduction

Although countless numbers of papers have appeared in the literature concerning the isolation and characterization of bacteriophages, relatively few of these have dealt with those bacteriophages involving the anaerobic bacteria, especially the genus Clostridium. A few phage isolations have been reported for this group of organisms but most reports are of a very preliminary nature. Most certainly no genetic studies have been conducted on these phages and only a very recent study of some bacteriophages active on Clostridium saccharoperbutylacetonicum has attempted a thorough examination of a clostridial phage from its isolation to its molecular biology. Many of the early reports of clostridial phages were concerned with "phagotherapy", or treatment with phages, of gas gangrene infections. These reports originated, for the most part, in Russia and very little information is available about the isolation of the phages and their characterization if, in fact, such studies were of interest at that time. It was, therefore, necessary to consult various abstracts of the Russian literature, one of which was the valuable bibliography of Spencer (1953).

Early Isolations : Bacteriophages of Cl. tertium and Cl. tetani

The first clostridial bacteriophage to be mentioned was that of Cl. tertium in 1931, when Cowles briefly mentioned its existence while discussing another but related subject. No previous reference to this phage was provided and no further description has ever appeared. Later, in 1934, Cowles described a bacteriophage for Cl. tetani in a paper which also proved to be a first and last report. This phage was isolated from crude sewage by an enrichment technique involving five strains of Cl. tetani. A lytic principle was obtained against two of the strains, one being more sensitive than the other. It was noted that by appropriate contact between the bacterial culture and the bacteriophage, the former could be made resistant and lysogenic, and cultures derived from heated spores of such a lysogenic culture contained bacteriophage.

The possible effect of bacteriophage on toxin production was of interest here and studies were performed to determine whether the presence of bacteriophage in a culture over a long period of time would alter the metabolism in such a way as to influence the production of either of the two tetanus toxins, tetanolysin and tetanospasmin. When no significant differences were noted Cowles concluded: "Presumably, therefore, the presence of bacteriophage cannot be held responsible for the poor toxin production

characteristic of some strains of Cl. tetani". This remark is of considerable interest since it suggested that toxin formation in bacteria might be repressed by bacteriophage, a suggestion which was realized in 1965 when Winkler, deWaart and Grootzen found that repression of Beta-toxin in Staphylococcus aureus could be brought about by bacteriophage.

Bacteriophages of Cl. perfringens and Other Clostridia Associated with Gas Gangrene

The isolation of bacteriophages active against Cl. perfringens, Cl. oedematiens, Cl. septicum, Cl. histolyticum and Cl. putrificus was successful by Russian workers in the early 1940's (Hauduroy, 1946; Zaeva et al., 1948). The presence of the bacteriophages was shown in many passages and in plaques on agar slides. Specific antibodies could be developed against these phages by immunization of rabbits. Such phages produced total lysis and were capable of destroying 96 per cent of the Cl. perfringens strains tested. This early work was also reviewed by Zhuravlev (1943) when he further described these phages which caused active lysis of the gas gangrene agents without harmful effects on the tissues of the body. Therapeutically, the phages were tested on guinea pigs and, prophylactically, they were successfully tested on wounded men (Treshchevskii and Grineberg, 1941; Zhuravlev, Kokin and Pokrovskaja, 1944). Intravenous and intradermal

applications of mixtures of phages specific for anaerobic and aerobic bacteria were found to be very successful in cases of gas gangrene, with mortality reduced to about one-third. Other Russian papers dealt with phagotherapy involving clostridial phages with somewhat similar results (Sirbiladze, 1942; Abesadze et al., 1944; Tseknovitser, 1944; Oleshkevich, 1946).

Tsyp et al. isolated bacteriophages for Cl. perfringens and Cl. oedematiens from sewage waters in 1944. The Cl. perfringens phages lysed type C organisms, but other types were not easily lysed.

A much more recent Russian paper has dealt with an electron microscopic study of a Cl. perfringens phage and its development within the bacterial cell (Bychkov, 1964). This phage had a structureless spherical head, 50-60 mμ in diameter, and a short tail, 15-20 mμ in length. Forty-five to fifty minutes after infection, the cell lysed with a discharge of up to 500 phage particles, which for some time remained adsorbed to the cytoplasmic remnants of the bacterial cells.

The second group of papers dealing with bacteriophages of Cl. perfringens originated in France. In 1947 Kréguer, Guélin and LeBris were successful in isolating a bacteriophage active on Cl. perfringens type A from sewage. Only one of the four type A strains tested was sensitive to this phage. Three other species of Clostridium showed no sensitivity to the phage.

Guélin (1949) continued the above studies, but found that this phage, designated as T, appeared difficult to handle with its low titers and incomplete lysis of the host organism. Therefore, another phage, M, was found and this phage produced high titers and caused complete lysis. A latent period of 15-20 minutes was shown to exist followed by a rising titer of infectious particles until 60-90 minutes after infection, at which point the titer fell. Complete lysis of the host organism occurred by 100 minutes after infection while the titer at its maximum was 34,500 times that of the input titer. Guélin noted that a propagation system employing large numbers of bacteria and small numbers of phage gave a poor yield, and when a large number of phage was used, a large number of bacteria was necessary for the maximum yield. She also noted that the lower the multiplicity of infection employed in such a system, the greater the increment in phage titer.

As with Cl. tetani, it was of interest to see if the bacteriophage had any effect upon toxin production in Cl. perfringens. For this study Guélin and Kréguer (1950) utilized a phage designated as 80b, which was active on Cl. perfringens type A-Weinberg. When phage was added to such a culture there was no noticeable alteration in toxin production.

In another study Guélin (1950 b) reported the presence of large numbers of Cl. perfringens phages in river water and

sewage and, after an attempt to determine whether the phage played a role in water purification, came to the conclusion that clostridial phages might be a potential agent in water purification but, that under the natural conditions of such an environment, phage multiplication was unlikely. Guélin (1950 a) also noted that the normal rod-shape structure of the clostridial cells was often replaced by swollen forms or spheres. These structures were not spores but were observed in phage infected cells as well as old cultures of non-infected cells. It was finally concluded that this phenomenon appeared to be the result of a non-specific reaction of the bacteria brought on by cellular irritation by different external agents, one of which was bacteriophage. A further study of peculiar granular forms associated with phage filtrates of Cl. perfringens by Guélin in 1953 was inconclusive.

The first electron microscopy on clostridial phages was performed by Elford, Guélin, Hotchin and Challice in 1953. Two Cl. perfringens phages were studied, M and W. Phage M possessed a head diameter of 35 mμ and tail dimensions of 120 x 15 mμ; phage W had a head diameter of 60 mμ and a tail similar to that of the M phage.

The activity of an antiviral drug, phagolessin (Hall and Asheshov, 1953), on six Cl. perfringens phages was determined by Levaditi, Guélin and Vaisman in 1953. This drug, obtained

from a Streptomyces species and acting on free phage, was found to inhibit two of the phage strains, but have no effect on the other four.

Also in 1953, Sames and McClung reported on the isolation of eight phages active on Cl. perfringens. Guélin (1955), still pursuing a toxin relationship with bacteriophage, demonstrated that normally toxin producing bacteria infected with phage were no longer capable of causing gas gangrene when injected into guinea pigs, while the bacteria alone produced the disease symptoms. This work further substantiated the Russian investigations on phagotherapy. In 1956, McClung briefly reviewed the clostridial phage literature.

One of the largest surveys of Cl. perfringens bacteriophages is to be found in the Ph. D. thesis of R. W. Sames (1956), who was working under the direction of L. S. McClung at Indiana State University, Bloomington, Indiana. This work described the isolation of 33 bacteriophage strains which could be divided into three serological groups and four plaque types. Burst sizes for these phages ranged from 150 to 1900 as described in one step growth experiments. A number of characteristics of five different phages was studied such as ultra violet light sensitivity, thermal inactivation, latent period, lowest pH of lysis, pH of maximum stability and susceptible bacterial strains. Although some of the plaque mor-



phologies might suggest the existence of a temperate bacteriophage, lysogeny was not observed in this experimental work. Unfortunately, none of this work has been published.

In 1959 Smith investigated lysogeny in Cl. perfringens and, in studying types A, B, C, D, E and F for phage relationships, found 12/49 A strains, 10/31 B strains, 10/26 C strains and none of 38 D, 5 E or 3 F strains were lysogenic. It was shown that the temperate phages were only active on strains of the same type from which they were isolated. Virulent phages were also isolated which lysed strains belonging to types A, B, C, D and F, although a high proportion of bacterial strains remained insusceptible to all the phages isolated. Propagation of the phages found on their respective indicator strains ruled out the possibility of lysis due to bacteriocins, which, incidentally, have been isolated and studied by Tubylewicz (1965, 1966). Induction of the lysogenic strains was successful with ultra violet irradiation, nitrogen mustard and thioglycollate. This is the only paper which has reported the existence of temperate bacteriophages of Cl. perfringens and the state of lysogeny. No characterization or description of the phages was made.

Gáspár and Tolnai (1959) performed one of the first informative characterizations of a virulent Cl. perfringens phage when they studied one of Guélin's phages designated as 808/3a.

A device was designed in this experiment for performing one step growth curves of the phage whereby the phage-bacteria culture was constantly agitated with bubbling nitrogen and samples were removed at various times for plating. A modified Wilson-Blair medium was employed for plaque counts and a thick overlay of agar containing phage and bacteria was used to produce anaerobic conditions without the aid of an anaerobic jar. The latent period of this phage was 45 minutes and the burst size, 452. Adsorption and immunological experiments were also conducted. In a second paper (Gáspár, 1960) relating the effect of aeration upon phage production, it was shown that aeration of the culture any time up to 45 minutes after infection prevented phage multiplication, whereas aeration at 45 minutes had no marked effect on phage production. Aeration of the culture at the time of infection greatly prolonged the latent period, the lag depending upon how long aerobic conditions were maintained. Aeration was shown to have no adverse effect upon the phage viability itself.

The most recent paper involving a bacteriophage of C1. perfringens has dealt with an electron microscopic study of Guélin's 1947 phage (Vieu, Guélin, and Dauguet, 1965). This phage was revealed as being 70  $\mu$  long with a polyhedric head 40  $\mu$  in diameter and a short tail 30  $\mu$  long. The tail portion was very unique in that it possessed what appeared to be a contracted plate

8  $\mu$  long and 34  $\mu$  in diameter containing 4 - 7 distinct structures. The tail core which projected beyond this plate was consistently demonstrated in all preparations.

#### Other Clostridial Phage Isolations

In view of the fact that the bacteriophages of Cl. perfringens were of greater interest to the author of this thesis, more time has been allotted to their discussion; however, a few other clostridial phages have been studied and these will now be reviewed. In 1940 Frenkel reported the isolation of a bacteriophage for Cl. sporogenes from river water. Media possessing a low redox potential were shown to demonstrate the best phage activity. No further work was reported on this species until 1963 and 1964 when Betz and Anderson isolated four bacteriophages for Cl. sporogenes from sewage and soil and made mention of the fact that McClung had also isolated a number of these phages from chicken faeces. These four phages plus eight of McClung's could be distinguished into three groups on the basis of their plaque morphology, host range, receptor sites and serological relationships. None of the 25 strains of Cl. sporogenes tested was shown to be lysogenic, although turbid plaque formation on some indicator strains suggest that the phage might be temperate in nature.

A report of four strains of bacteriophage in a butyl fermentation plant suggested the problem of phage in such an

environment was an undesirable one but the species of Clostridium was not mentioned (McCoy, McDaniel and Sylvester, 1944). A little more informative, however, was the report of Gold and Watson (1950 a) on a bacteriophage of Cl. madisonii. They found that the usual overlay method for plaque development on solid media was not satisfactory for a gas producing anaerobe, since the surface layer was disrupted by gas. This problem was evidently overcome or not mentioned by workers using Cl. perfringens. Therefore, a method was devised whereby the turbidity of bacterial growth in a molasses medium was the criterion of lysis caused by bacteriophage. By making appropriate dilutions of the phage an end-point of lysis could be determined which was related to the number of infecting particles. In a further study (Gold and Watson, 1950 b) the effect of pH on infection and lysis was determined.

Two clostridial species and their phages have been studied in some detail by Japanese workers. In a series of six papers Kinoshita and Teramoto (1955 a, b, c, d, e, f) have studied the bacteriophages of Cl. acetobutylicum. Three phages were described, one of which was temperate. When high concentrations of the temperate phage were plated with the bacteria, a uniform layer of bacterial growth was obtained, a fact which suggested lysogenization of the indicator strain. Plaques produced by this phage were obscure, while those produced by the other two phages were clear.

The sensitivity of the phages to heat, irradiation and nine chemicals was determined. Both in molasses and synthetic media the first burst occurred about 90 minutes after the addition of the phage with a burst size of 20. Calcium and magnesium ion concentration was important for rapid and high phage titers.

The temperate phage was shown to lysogenize its indicator strain and that when this now lysogenic strain was allowed to sporulate, heat treatment of the spores did not destroy their ability to produce phage when germinated. This temperate phage was also shown to cause "sluggish fermentation" of butanol, although no lytic activity could be observed.

A second group of Japanese workers has recently conducted a detailed study on twelve bacteriophages of Cl. saccharoperbutyl-acetonicum (Hongo and Murata, 1965 a; 1965 b). These phages, designated as HM phages, were isolated from an industrial plant where abnormal acetone-butanol fermentation was occurring. No lysogeny was detected in the bacterial strains tested. The twelve phages could be divided into three groups based on electron microscopy (Hongo and Murata, 1966 a), plaque morphology (Hongo and Murata, 1966 b), and serological relationships (Hongo, Aono and Murata, 1966). It was of interest to note that high centrifugal force (85, 230 g) used in the purification procedures of the phage lysates caused a drastic loss in phage viability, which was evidently due to loss or damage

of the tall structures (Hongo and Murata, 1966 a). A smaller centrifugal force (34, 850 g) was found to be satisfactory for centrifugation of viable phage particles in broth, while still smaller gravitational forces were employed for buffer preparations of the phage. These phages were relatively sensitive to temperatures over 50° C (Hongo and Murata, 1966 b).

A considerable number of experiments was performed in characterizing the phages and their growth conditions such as the effect of the bacterial inoculum size, agar depth and concentration, temperature and pH upon plaque size and morphology (Hongo and Murata, 1965 a); adsorption and one-step growth experiments (Hongo, Miyamoto and Murata, 1966); and phage stability to ultra violet light and various pH ranges (Hongo and Murata, 1966 b). Hongo, Miyamoto and Murata (1966) proposed that they were the first workers to perform a one-step growth curve on a clostridial phage and that bubbling nitrogen through the growth mixture was a new approach to such studies. These workers were evidently not aware of the French work on clostridial phages and that of Gáspár and Tolnai, who had devised a similar technique in 1959.

Of considerable significance in the Japanese work is the fact that Hongo, Ono, Ogata and Murata (1966) have made the first nucleic acid determinations recorded for a clostridial bacteriophage. A phenol extraction of nucleic acid from a purified

and concentrated phage preparation was performed and this extract was characterized by analytical ultracentrifugation, chromatography, thermal denaturation and chemistry. The phage nucleic acid, which was DNA, was shown to have no unusual bases and not to differ greatly from the DNA composition of the host bacteria, which was also studied in the same manner.

Both chloramphenicol and oxytetracycline were observed to repress the production of Cl. saccharoperbutylacetonicum phages in antibiotic resistant mutants of the bacteria (Murata and Hongo, 1964; Hongo, Ono, Kono and Murata, 1966).

One other paper to appear on clostridial phages discussed a bacteriophage isolated from one of three lysogenic strains of Cl. histolyticum (Guélin, Beerens and Petitprez, 1966). Only one of the 14 strains tested was sensitive to this phage. Adsorption of this phage appeared to be very slow when heat-killed cells were used as the substrate. Unfortunately this paper, like many of the earlier French papers, fails to present much detail of the experimental procedures involved; however, this paper does present some excellent electron photomicrographs of the phage with its 80 mμ polyhedral head and 240 mμ tail. Tail striation was obvious and an end plate 30 mμ in width was suggested by these pictures.

### Concluding Remarks

This review of clostridial phages has attempted to cover as many phage isolations as possible and to report some of the experimental results obtained in the studies involving these phages. Although the bacteriophages of Cl. perfringens were more intimately involved with the subject of this thesis, it was felt that so relatively little was known about these phages that a review of wider scope might be appropriate. Indeed, the literature and reports are so diversified and widely scattered concerning these phages that a gathering of such information is almost essential in evaluating the position of clostridial phages in relation to that of aerobic phages. Although such a comparison and evaluation is not the aim of this work, it is obvious from the literature that clostridial phages do not differ greatly from those of the aerobic bacteria either in morphology or growth characteristics. Both temperate and virulent phages have been reported, although reports on the former have been rare, perhaps due to a lack of interest in looking for lysogenic systems. It would seem, then, that only the anaerobic conditions required for the host bacteria differentiate the two systems. Whether the phage directed enzyme systems are strictly dependent upon anaerobic conditions when active within the host cell is not known, although Gáspár's paper



on aeration of infected Cl. perfringens cells would lead one to propose that such might be the case.

There is much, therefore, which could be done with clostridial phages in the future. Studies involving further characterization of these phages are certainly warranted and the fields of genetics and molecular biology are wide open for investigation.

## MATERIALS AND METHODS

### Origin of Clostridial Strains

The strains used in this study were obtained from Dr. V. Fredette, Institute of Microbiology and Hygiene, Laval-des-Rapides, P.Q.; Dr. L. S. McClung, Indiana State University, Bloomington, Indiana; The Department of Microbiology and Immunology and the Clinical Laboratory of the Royal Victoria Hospital, McGill University, Montreal, P.Q.; the American Type Culture Collection; and one isolation was made from waters surrounding Montreal. These strains are listed in Table I. The personal code established for these strains will be used in the text of this thesis.

### Identification of *Cl. perfringens* Strains

Although the strains collected had been identified by others as *Cl. perfringens*, all were rechecked for characteristic features of the species. The more important features noted were smooth, entire, raised and circular colonies demonstrating haemolysis on sheep blood agar; lecithinase production on egg yolk agar; stormy fermentation of litmus milk with acid production; fermentation with acid and gas production in Hiss serum sugars containing glucose, lactose, maltose, sucrose, but not fermenting salicin; microscopically, the presence of large Gram-positive rods occurring singly, or more

TABLE I

Source of Cl. perfringens Strains

Personal Code	Original Code	Type	Strain	Source	Origin
1		A		McGill	Teaching Strain
2	677	A	Q21-S	McClung	G.B.Reed Collection
3	686	A		McClung	Cooked chicken
4	694	A	117-A	McClung	Chicken broth
5	695	A	122	McClung	Raw chicken
6	1156		10-a	McClung	Cow faeces (Sweden)
7	T1			Fredette	Soil
8	T9			Fredette	Soil
9	T12			Fredette	Soil
10	F4			Fredette	Faeces
11	F7			Fredette	Faeces
12	F8			Fredette	Faeces
13	Lechien			Fredette	Standard French strain
14	28 mutant			Fredette	
15	SWG 121			Fredette	Standard Canadian strain
16	SR 12			Fredette	Standard American strain
17	21051			McGill	Clinic : cervix uteri
18	Lachine			Mahony	River water
19	680			McClung	
20	3626	B		A.T.C.C.	Lamb Intestines
21	3627	B		A.T.C.C.	Heart Blood of Lamb
22	41681			McGill	Clinic (Ulcer swab)
23	2351			McGill	Clinic (Labial abscess)

frequently, in pairs and not bearing spores; and absence of growth under aerobic conditions (Bergey's Manual of Determinative Bacteriology, 7th Edition). Strain 9 differed from the others in the absence of haemolysis on sheep blood agar.

### Maintenance of Strains

#### Lyophilization

After assuring the purity of the various strains, they were grown on blood agar slopes for 24 hours, washed free in a suspending fluid composed of broth, glucose and serum (Fry, 1951) and lyophilized.

#### Brewer's Meat Cultures

The strains in use during a series of experiments were kept in cooked meat broth. (See Appendix I). Stock cultures were kept at 4° C, a temperature much more suitable than room temperature for the preservation of these organisms, and subcultures were made from these when necessary. This procedure was adopted after it was noted that repeated transfer of the organism in cooked meat appeared to detrimentally influence its growth on Lab Lemco agar (see below), and prolonged viability at room temperature was not dependable.

### Bacteriophages

Two virulent bacteriophages of Cl. perfringens, designated as Cp 2 and Cp 7, were obtained from Dr. L. S. McClung. Their indicator strain (McClung No. 680) was also provided.

### Media

#### Solid Media

In order to observe plaques formed on a confluent lawn of bacterial growth, a clear medium must be employed. Four media were tested for their support of bacterial growth: sheep blood agar (as a control for good growth), Lab Lemco agar, Lab Lemco agar containing 0.2% sodium thioglycollate and Fildes' agar. After 18 hours of anaerobic incubation, good growth was obtained on all these media with both spread plates and semi-solid agar overlay preparations. Since Lab Lemco agar was readily available within the Department, this medium was chosen for the basal layer in propagation of the phages on solid media. The organism did not produce observable gas when grown on this medium, an essential quality when a semi-solid agar overlay is used for plaque titrations. The preparation of this medium is described in Appendix I.

#### Semi-solid Agar

The soft agar overlay used in seeding the surface of Lab Lemco agar plates with bacteria was prepared by boiling a

0.6% solution of Noble Agar (Difco Laboratories, Detroit, Michigan) in distilled water and distributing the melted agar in 2.5 ml amounts into small metal-capped tubes. These were sterilized for 20 minutes at 121° C and subsequently stored at 4° C until used. For each experiment an appropriate number of tubes was melted by boiling and then cooled to 45° C in a water bath until used.

### Liquid Media

#### Broth

Dehydrated Brain Heart Infusion Broth (Difco Laboratories, Detroit, Michigan) was resuspended in distilled water as prescribed by the manufacturer and 0.1% sodium thioglycollate (Baltimore Biological Laboratories) w/v was added. This medium, subsequently referred to as "broth", was sterilized for 20 minutes at 121° C and stored at room temperature. The broth was always boiled and cooled prior to inoculation to remove dissolved oxygen.

#### Brewer's Cooked Meat

This medium was used for the maintenance of the bacteria as well as for initial growth prior to subculturing to broth in the experimental work. This medium was also boiled and cooled before inoculation. The details of preparation are presented in Appendix I.

### Incubation Techniques

All cultures were incubated at 37° C unless otherwise stated. Plate cultures were incubated in an anaerobic incubator (National Appliance Company, Portland, Oregon). The chamber was evacuated to a pressure of 360 mm of mercury and then filled with hydrogen until a pressure of 735 mm of mercury was obtained. Six platinum-palladium dry catalysts were kept in the incubator to promote the reduction of the remaining oxygen to water. Incubation was carried out for 18-20 hours. For a small number of plates, Brewer's anaerobic jars were used.

### Assay of Phage by the Agar Layer Method

A modification of the method described by Adams (1959) was employed. Phage preparations were diluted appropriately in broth, of which 0.1 ml was placed on the center of an agar plate. Approximately 0.5 - 0.8 ml of a three hour indicator culture was added by Pasteur pipette to 2.5 ml of molten semi-solid agar at 45° C. The semi-solid agar tube was briefly mixed and the contents poured on top of the applied phage sample. Mixing of the phage and bacteria was effected by gently rocking the plate, causing an even distribution of fluid over the entire surface. By adding the phage sample directly to the plate rather than mixing it with bacteria in the warm agar, one assures that the total phage sample

is delivered to the plate. An excellent distribution of plaques was always obtained by this method.

#### Ultraviolet Irradiation Experiments

Two 15-watt General Electric (G15 T8) germicidal lamps were used as a source of u.v. irradiation. The distance from the light source to the irradiated surface of Petri plates was 38 cm. Plates containing appropriate suspensions of bacteria or phage were slowly rocked by hand beneath the light source for various time intervals, after which the glass lids of the plates were replaced and covered with aluminum foil until sampling was performed. An effort was made to reduce the lighting conditions of the room during all u.v. experiments.

#### Chemical Methods

All chemicals used in the experimental work were of reagent grade.

#### Nucleic Acid Extraction

Phage and bacterial nucleic acids were extracted by cold and hot perchloric acid (PCA) treatment of the test materials. The procedure, which is presented in Appendix II, is a modification of the Schneider, Hogeboom and Ross technique (1950).



## Nucleic Acid Determination

### Deoxyribonucleic Acid (DNA)

DNA was determined by the Burton modification of the Dische method (Burton, 1956), a colourimetric test using diphenylamine, aqueous acetaldehyde and perchloric acid. Details of the procedure and chemicals involved are presented in Appendix II.

### Ribonucleic Acid (RNA)

RNA was determined by the Dische modification of Mejbaum's method (Ashwell, 1957), a colourimetric test using ferric chloride-containing hydrochloric acid and orcinol. The details of the procedure appear in Appendix II.

## Collection of Water Samples

### Apparatus

An attempt to isolate bacteriophages for Cl. perfringens from the waters surrounding the city of Montreal was made. In order to collect such samples, equipment had to be designed.

An eight-foot fishing rod, with reel containing 150 feet of nylon fishing line, was purchased. Two carriages were built to hold the collecting vessels, one of plastic and capable of holding a four-ounce screw-cap bottle, and the other of wire and

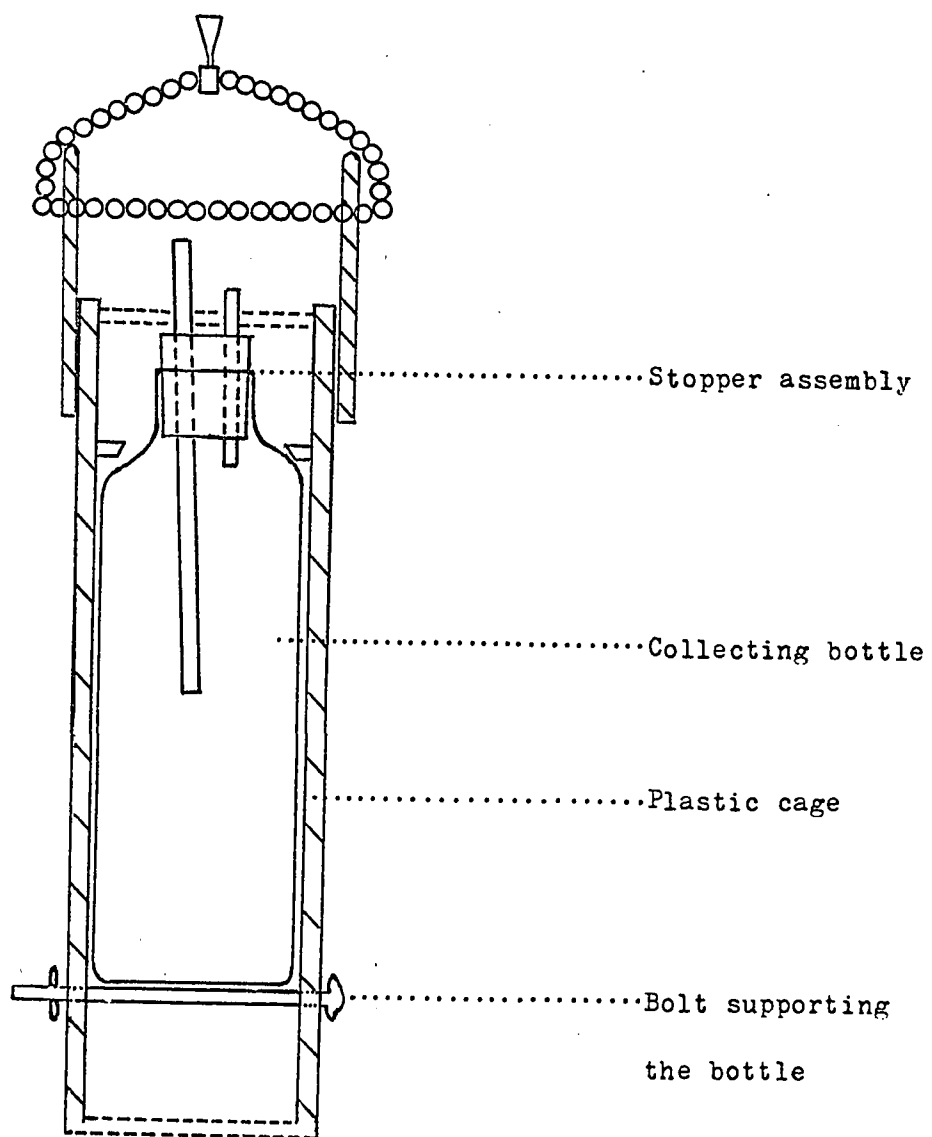


FIGURE 1: Diagram of water collecting apparatus.

tin capable of holding a 18 x 150 mm test tube. The weight of each loaded container was sufficient to sink the apparatus in water. A No. 2 rubber stopper (which fit both vessels) was penetrated by two pieces of glass tubing, one piece being shorter than the other and serving as a water inlet (See Fig. 1). Such a stopper allowed easy and rapid filling of the vessels when lowered below the water's surface. The fishing line was marked at one-foot intervals so that the approximate depth of the sample could be noted. Judging by air bubble disturbance below the surface, the filling of the bottle or tube did not occur before it had completed its descent.

#### Water Sampling and Plating

Water samples were taken in two amounts - 30 and 140 ml. When returned to the laboratory 0.5 ml of each sample was spread over the surface of two sheep blood agar plates and allowed to dry; one plate was incubated aerobically and the other anaerobically. This screening method served only to provide some idea of the relative number of bacteria present in any one sample. Colonies resembling those of Cl. perfringens on the anaerobic plates were subcultured.

#### Enrichment Technique for Phage Isolation

Water samples were filtered through 20 mm Sartorius membrane filters which had a mean porosity of 0.15  $\mu$  (Carl Schleicher

and Schuell Company, Keene, New Hampshire). Five ml of the fluid passing through the filter was added to a  $2\frac{1}{4}$  hour fluid culture containing a mixture of 17 strains of Cl. perfringens, and incubation was continued for three hours. This culture was then centrifuged to remove bacterial cells and the clear supernatant fluid was assayed for phage against the 17 strains individually.

#### Assay of Water Samples for Phage

The clostridial strains used as prospective indicators were grown for 6-8 hours in broth. To air-dried agar plates were added 1.5 ml volumes of culture which were spread over the surface, the excess fluid being withdrawn with a Pasteur pipette. After the surface of the plates appeared dry, one drop of the water sample was allowed to run across a section of the plate. Five different samples could be applied to one plate.

#### Ammonium Sulphate Precipitation of Phage

Ammonium sulphate (about 400 g) was used to saturate 500 ml of pooled water samples. After standing at 5° C overnight to allow protein flocculation, the surface scum was removed and resuspended in 50 ml of broth. Five ml of this suspension was added to 50 ml of a six hour enrichment culture and incubated overnight. After centrifuging to remove bacteria, the supernatant

fluid was assayed for phage. This method was described by Sames (1956).

#### Other Materials and Methods

A number of other techniques and materials were employed throughout this study and are described in context with the experimental results.

## EXPERIMENTAL PROCEDURES AND RESULTS

### Attempts to Isolate Bacteriophages

#### Failure to Detect Phage in Water

Thirty-six water samples were collected from several locations in and around the Montreal area. After filtration and enrichment procedures, these samples were cross-streaked against 17 available strains of Cl. perfringens, as described in Materials and Methods.

Although what appeared to be plaques from time to time was picked to the respective indicator strain, no propagation occurred and plaques could not be reproduced. These plaques were, therefore, considered artifacts caused, perhaps, by the plating technique. It was found that the growth of the "indicator" strains on agar plates was better when six to eight-hour cultures were used rather than overnight cultures.

Only one strain of Cl. perfringens was isolated from the water samples. Since no bacteriophages were isolated from the water samples, the bacteriological findings in such specimens will not be discussed.

#### Cross-streaking for Lysogeny

When no phages were found in the water samples, strains 1 - 17 of Cl. perfringens were cross-streaked against each other

in an attempt to detect a lysogenic strain. Two ml of an 18 hour culture was added to the surface of agar plates and, after removal of excess fluid with a Pasteur pipette, the plates were dried at 37° C for 30 minutes. After each strain was so plated, a drop of culture from a Pasteur pipette was allowed to run across the seed layer of each plate. Five different cultures could be applied to one plate.

No plaques were observed, although a few suspicious looking areas were picked without success.

#### Assay of Supernatant Fluids for Phage

Fourteen-hour cultures of strains 1-18 were centrifuged to remove the bacterial cells and the supernatant fluids were spotted onto a semi-solid seed layer containing a 7½-hour culture of Cl. perfringens. A multiple inoculator previously designed was used for this purpose (Mahony, 1965). No plaques were observed; however, it was found that areas of clearing often occurred in the semi-solid layer at the site of inoculation. These were shown to be artifacts due to the mode of inoculation.

#### Sensitivity of the Bacteria to Ultraviolet Light

Prior to induction experiments with u.v. light, an estimate of the sensitivity of the clostridial strains to irradiation was

made. Abnormal sensitivity might indicate the presence of a lyso-genic strain.

Ten-hour 8 ml cultures of 18 strains were centrifuged to sediment the bacterial cells. The resulting supernatant fluids were discarded; the sedimented bacteria were washed twice in 0.85% saline; resuspended to the original volume in saline; and were exposed to u.v. light for 1, 2, 4, 6 and 8 minute intervals. After each irradiation period a 0.1 ml sample of cells was spread over the surface of blood agar plates and these were then incubated. When the irradiations were completed, the bacteria were again centrifuged and resuspended in broth and incubated for four hours, during which period the cultures were observed for lysis.

Twelve strains showed a marked inhibition of growth after one minute, five after two minutes and one strain after four minutes of irradiation. Strains 1, 2, 13 and 16 appeared more resistant to u.v. irradiation. No lysis of the cultures was observed. It thus appeared that an inducing dose should not exceed two minutes of irradiation, since death of the bacteria was not due to lysis.

#### Assay of U.V. Irradiated Culture Supernatant Fluids

Eighteen strains of Cl. perfringens were prepared for irradiation as described above. An irradiation of 100 seconds



was allowed, after which the bacteria were centrifuged, the supernatant fluid was decanted, and the sedimented cells were resuspended in broth for a four hour incubation period. These cultures were then centrifuged and the supernatant fluids were assayed for phage. The multiple inoculator was again used to place a small drop of these broths onto a semi-solid layer containing an indicator strain.

#### Isolation of a Bacteriophage

A single small plaque was produced by the supernatant fluid of strain 9 spotted on strain 13. This plaque was picked with a sterile inoculating wire into 1.5 ml of broth containing 7-8 drops of a 12-hour culture of strain 13. The culture was incubated overnight under anaerobic conditions and then centrifuged. A 0.1 ml amount of the supernatant fluid was assayed using the semi-solid agar overlay method and strain 13 as the indicator organism. Many plaques were found distributed over the surface of the plate after overnight incubation. Several of these plaques were picked to another 1.5 ml amount of culture and the propagation was repeated. Plating of a 0.1 ml sample of this second cycle of propagation produced confluent lysis of the indicator organism. Thus, there was no question about the presence of a phage specific for strain 13, since multiplication of the phage was occurring with both liquid and solid media.

### Description of Plaques

The plaques produced by this phage were small, and varied from pinpoint to 1.5 mm in diameter. With the larger plaques a turbid center was readily observed while the smaller ones only showed the same characteristic when stained. (See Staining Technique for Bacteriophage Plaques). The edges of the plaques were not entire but rather ragged. Picking single plaques of contrasting size and propagating such examples before plating again resulted in the same variation in plaque size when replated. This suggested that only one phage type was present and that the variation in plaque size might be due to the depth in the agar overlay or perhaps due to lysogenization of the indicator strain with subsequent release of phage at varying times.

### Comparison with McClung Virulent Phages

The two virulent phages (Cp2 and Cp7) and their indicator strain 19 were kindly provided by Dr. L.S. McClung. Upon receipt these preparations had titers of 5.8 and  $9.7 \times 10^8$  plaque forming units (PFU) per ml, respectively. The plaques, about 1 mm in diameter, were clear and after the plates were kept for four days at room temperature on the bench, the plaque size increased up to 5 mm in some cases. This was probably due to an enzymatic lysis of surrounding cells.

To two-100 ml volumes of broth was added 1 ml of a six-hour culture of the indicator strain. One hour of incubation at 42° C (recommended by McClung) produced slight turbidity and, at this point, about  $10^7$  PFU of each McClung phage was added to the two cultures respectively. Complete lysis of the cultures occurred within 1-2 hours of further incubation.

With the temperate phage isolate, no apparent lysis of the culture ever occurred and titers rarely exceeded  $2 \times 10^8$  PFU/ml. The host range of the phages differed as follows: Cp2 was active on strains 2, 3, 5, 11 and 19; Cp7 was active on strains 2, 3, 11 and 19; the temperate phage was active only on strain 13.

In view of the failure to isolate bacteriophages for Cl. perfringens from polluted waters, no further attempts were made to isolate virulent phages. With the successful isolation of a temperate phage from a lysogenic strain of Cl. perfringens and the detection of an indicator strain for this phage, specific attention was focused on temperate bacteriophages and lysogeny. Such a move was prompted by the fact that a temperate bacteriophage for Cl. perfringens had never been described in the literature, although the existence of such phages was demonstrated by Smith in 1959.

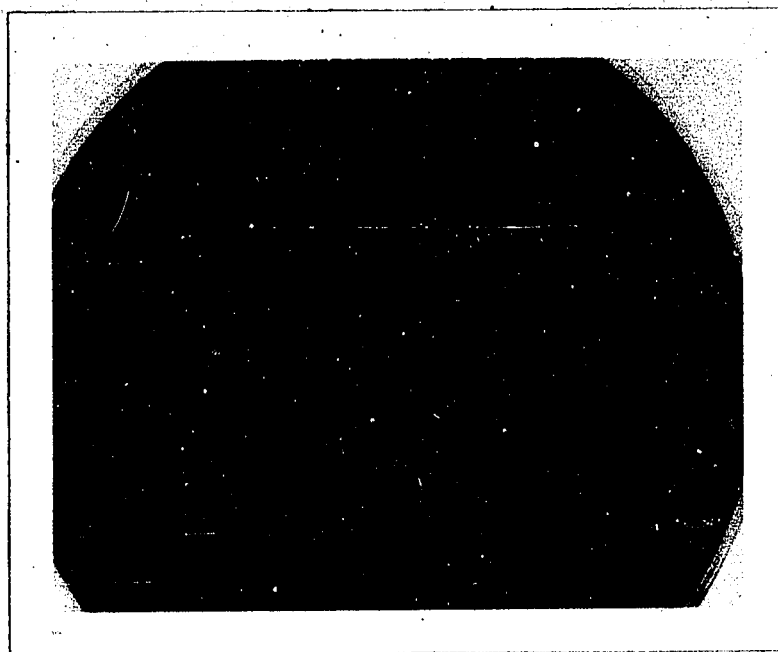
The experiments which follow were thus devoted to the characterization of this temperate bacteriophage and the nature of lysogeny.

### Staining Technique for Bacteriophage Plaques

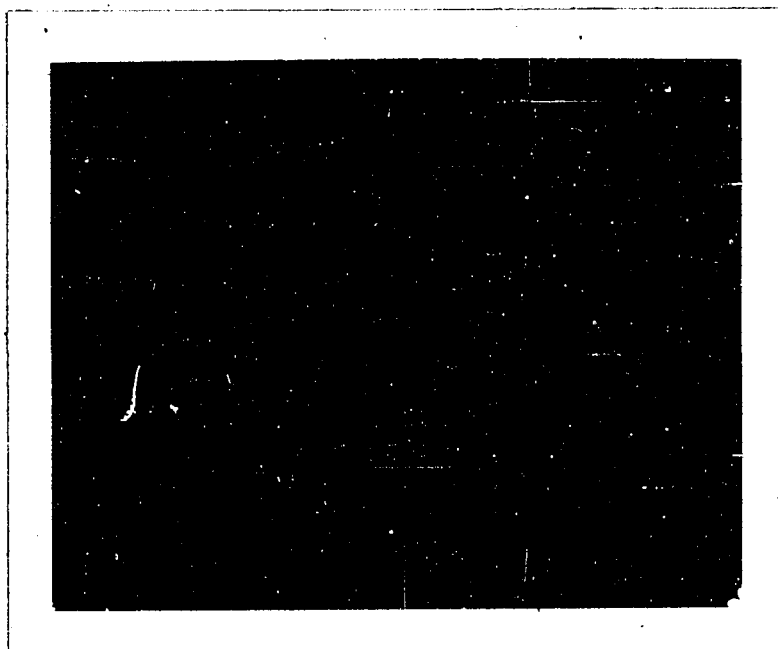
The plaques formed by the phage on solid medium were always small and often difficult to see. In order to photograph these plaques, it was desirable to stain the plaque bearing plates in some differential manner to provide contrast between the plaque and the lawn of bacterial growth.

A very simple method was devised for this purpose which utilized the common stains of the Gram staining technique. The surface of an agar plate bearing plaques was flooded with dilute carbol fuchsin for 30 seconds and then the dye was decanted from the plate. Absolute ethanol was subsequently used to remove excess carbol fuchsin by flooding the plate with this agent and washing the surface by tilting the plate back and forth for 20 seconds, at which time the alcohol was decanted. The plate was next flooded with Gram's crystal violet and tilted back and forth until the dye penetrated the surface of the agar; when stained, the dye was decanted from the plate and the surface was air dried in an incubator at 37° C for one or two minutes.

The stained plates, when observed with transmitted light, revealed a purple lawn of bacterial growth with sharply defined, unstained plaques. The plaque morphology was not altered in any observable way. Fig. 2a depicts typical plaque morphology as produced in the semi-solid agar overlay technique.

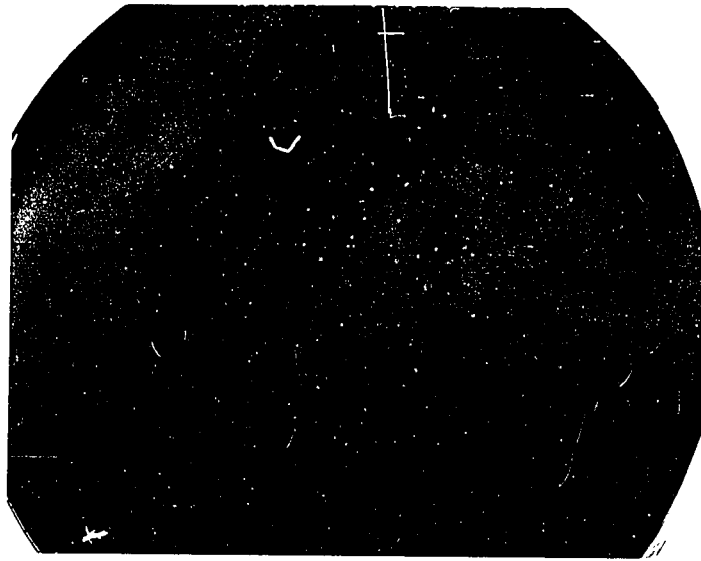


(a) Appearance of plaques on a stained plate. (1.17 x)

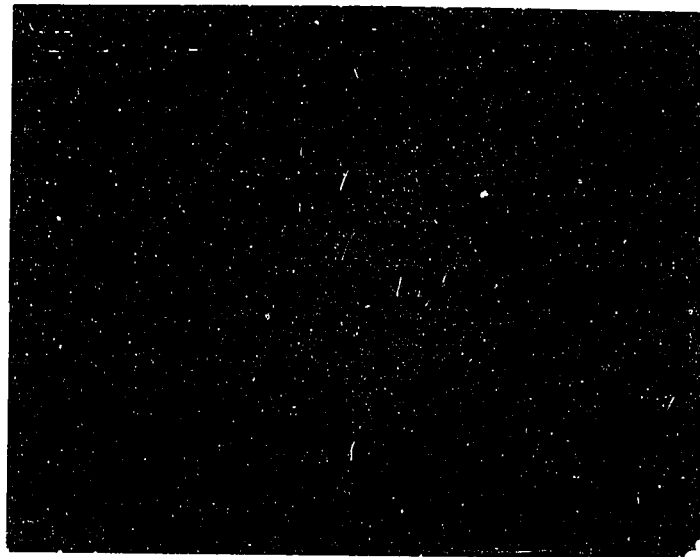


(b) Turbid plaque (31 x)

FIGURE 2: Staining technique for bacteriophage plaques.



(a) Appearance of plaques on a stained plate. (1.17 x)



(b) Turbid plaque (31 x)

FIGURE 2: Staining technique for bacteriophage plaques.

The photograph was taken with a Polaroid MP-3 camera utilizing an Ednalite CTB-1 (82) filter. The lens opening was f/11 and the exposure time was one second.

Fig. 2b portrays a turbid plaque as viewed through a Zeiss microscope employing a 2.5x objective lens and a KPL 12.5x eyepiece. A white, ground glass filter was used to provide diffuse transmitted light.

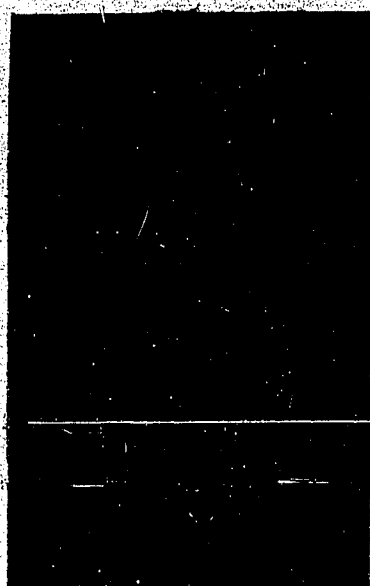
#### Electron Microscopy

The electron micrographs of the temperate bacteriophage of Cl. perfringens were prepared by Dr. R.G.E. Murray, University of Western Ontario, London, Ontario. The preparations were made with neutralized (pH6.8) phosphotungstic acid. The phage was supplied in phosphate buffer containing  $2 \times 10^{-3} \text{ M MgSO}_4$ , having been partially purified by centrifugation in a Model L Spinco ultracentrifuge under a centrifugal force of 78,000 g (Rotor No. 30) for two hours.

These pictures revealed that the bacteriophage had a very regular hexagonal head, probably representing an icosahedral capsid, measuring  $800 \text{ \AA}$  long and  $700 \text{ \AA}$  wide; the tail structure of the phage was about  $2000 \text{ \AA}$  long and  $80 \text{ \AA}$  wide (see Fig. 3 a,b). Most of the bacteriophage heads in the preparations appeared to have lost their nucleic acid (Fig. 3 d), perhaps because of the purification technique.

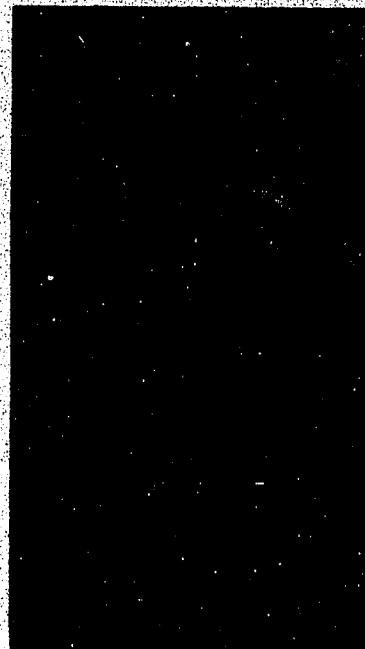
The tail was striated with a striation frequency of  $40 \text{ A}^{\circ}$ , as is best shown in Fig. 3 c. Fig. 3 d demonstrates what appears to be a small collar or plug at the end of the tail where the head has become detached. No evidence of an end-plate, tail fibers or contractile sheath was provided in these pictures, although the technique employed in the preparation of the specimens was not considered as being too suitable for the preservation of tail fibers. However, the tail morphology of this temperate phage is in keeping with that of most temperate phages which have been studied, these phages apparently possessing a much simpler tail structure than that of the virulent phages.



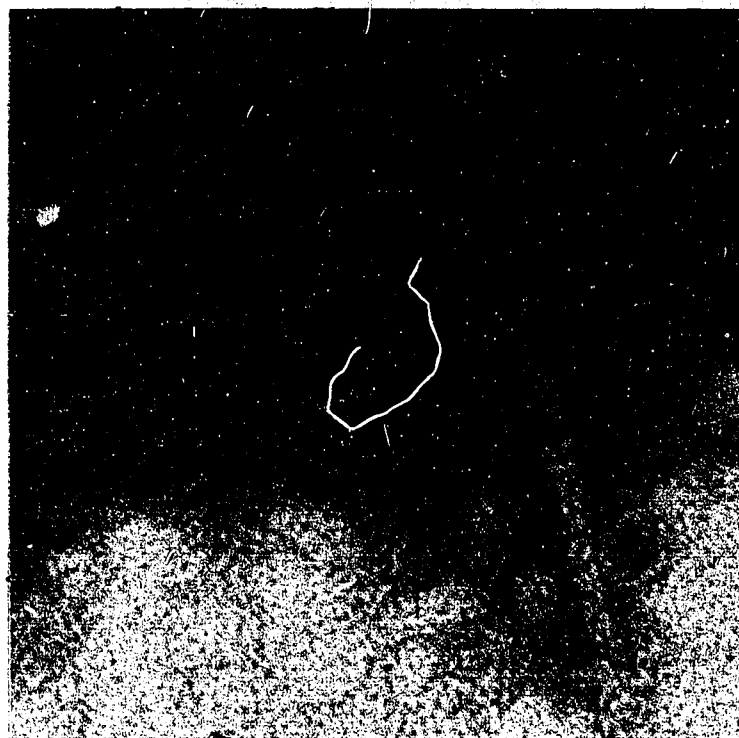


(a) 1000 Å

59 a



(b) 1000 Å



(c) 1000 Å

FIGURE 3: Electron photomicrographs of the temperate bacteriophage of Cl. perfringens.



(a)  $1000 \text{ \AA}$



(b)  $1000 \text{ \AA}$

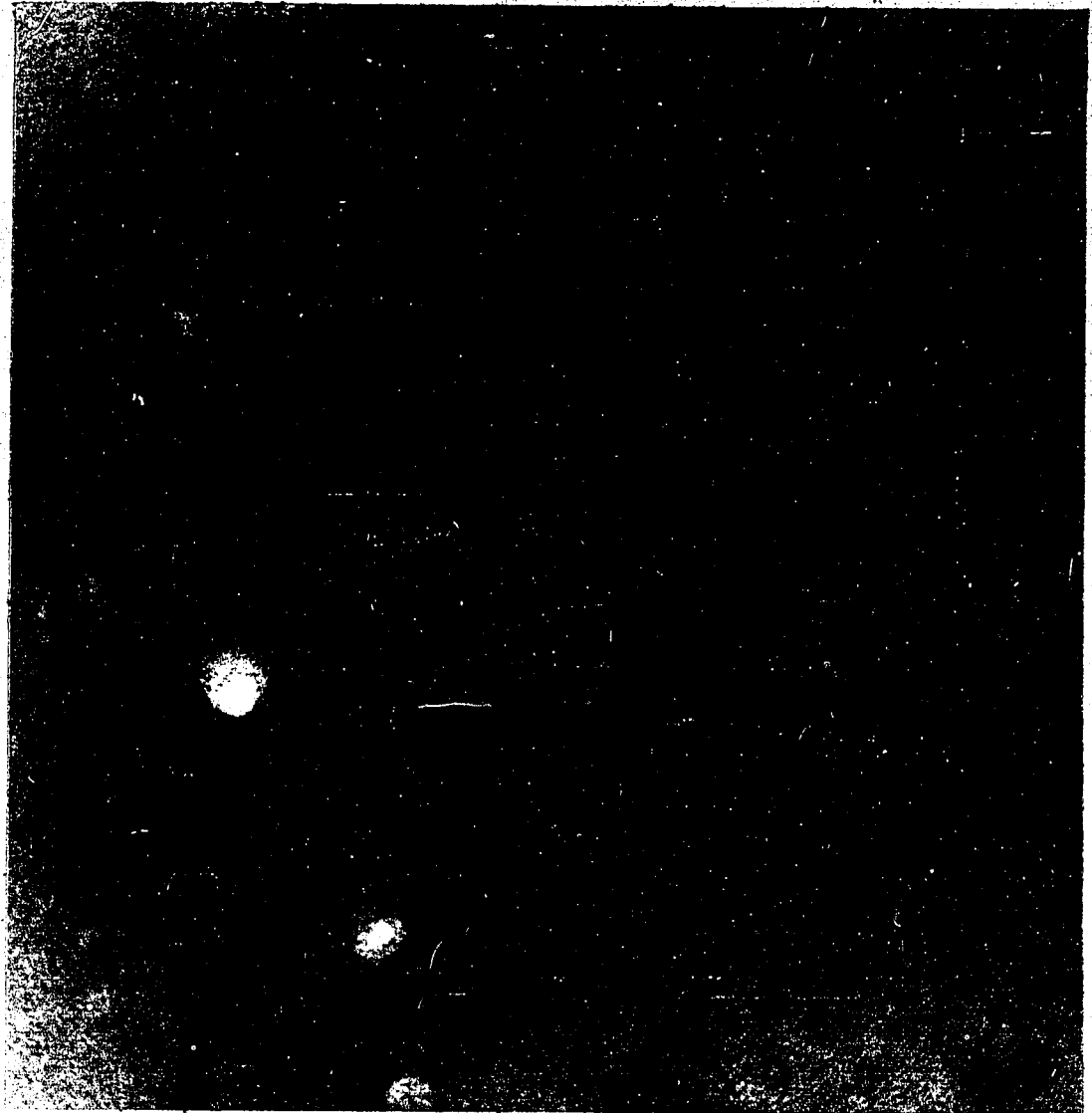


(c)

$1000 \text{ \AA}$

FIGURE 3: Electron photomicrographs of the temperate bacteriophage of Cl. parvulus.

59 b.



(a)

1000 Å



1000 Å

Growth Studies on the Indicator Strain, 13Growth at 37° C and 41° C as Determined by Optical Density

It was found that Pyrex glass tubes 18x150 mm (Corning Glass Works, Corning, New York) were virtually optically identical when examined in a Bausch and Lomb "Spectronic 20" spectrophotometer. New washed tubes were used in these experiments.

A few drops of an overnight culture of strain 13 were added to 15 ml of broth in one of the above tubes in order to produce an optical density (O.D.) reading of 0.12 at 660 mμ in the Spectronic 20, which contained a red filter and phototube for transmission at this wavelength. A tube containing broth was used as a blank. Incubation was performed at 41° C in a water bath and O.D. readings were made at various times. A parallel experiment utilizing an incubation temperature of 37° C was also performed. The initial O.D. of this culture (0.08) was slightly lower than that of above culture.

The results are recorded in Fig. 4. The growth of this organism was very rapid with lag plus logarithmic phases not exceeding  $3\frac{1}{2}$  hours. There appeared to be very little difference between the O.D. readings of these two growth curves, suggesting that either 37° C or 41° C might be used as incubation temperatures without appreciably altering the growth pattern of this organism.

60 a

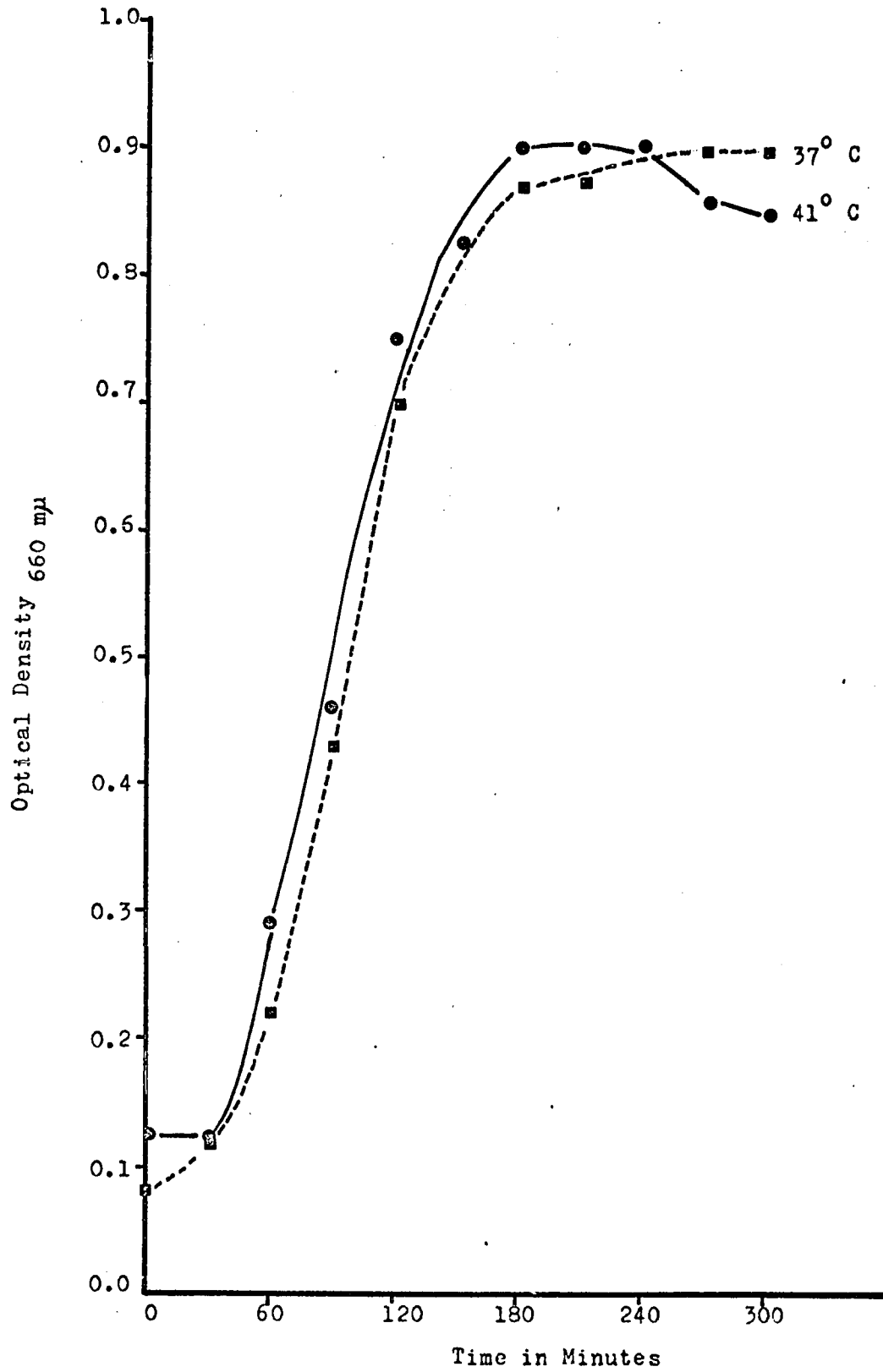


FIGURE 4: Growth of *Cl. perfringens* at 37° C and 41° C.

McClung has suggested the use of the higher temperature for the growth of Cl. perfringens.

#### Viable Count and Optical Density

Two tubes containing 15 ml of broth each were inoculated with 0.6 ml of an overnight culture of strain 13. An uninoculated broth served as a blank. Both culture tubes were stoppered and incubated at 37° C, and O.D. 660 mμ readings were made at 30-minute intervals. From one of the tubes were taken 0.1 ml samples every 30 minutes. The other stoppered tube was a control which served to determine whether the sampling procedure in the first tube interfered with the normal growth of the organism.

An appropriate dilution of the 0.1 ml samples was made in chilled broth and 0.1 ml amounts of this dilution were plated in duplicate on blood agar plates. A wire spreader was used to distribute the bacteria evenly over the surface of the plates. All inoculated plates were refrigerated until completion of the sampling, at which time they were incubated anaerobically.

The results are recorded in Table II and Fig. 5. The viable count reveals a lag of about one hour, then a short logarithmic phase of two hours followed by a rather rapid decline in viability. After three hours of incubation the pH of the culture was 6.5. The removal of samples from the culture tube did not appear to interfere with the growth curve as determined by O.D. readings.

TABLE IIViable Count and Optical Density Growth Curves of Strain 13

Time (Min)	Viable Count/ml $\times 10^7$	O.D.
0	2.1	0.08
30	2.6	0.12
60	2.6	0.22
90	9.2	0.43
120	19.0	0.70
150	35.7	0.83
180	52.5	0.87
210	43.5	0.88
270	21.5	0.90
300	19.0	0.90
360	12.8	0.86

From these data, it appears that the entire cycle of growth for this organism is very short and that such a culture should probably be infected at an early stage if the logarithmic phase of the bacterial growth is to be utilized for phage production.



62 a

■ viable count  
● optical density

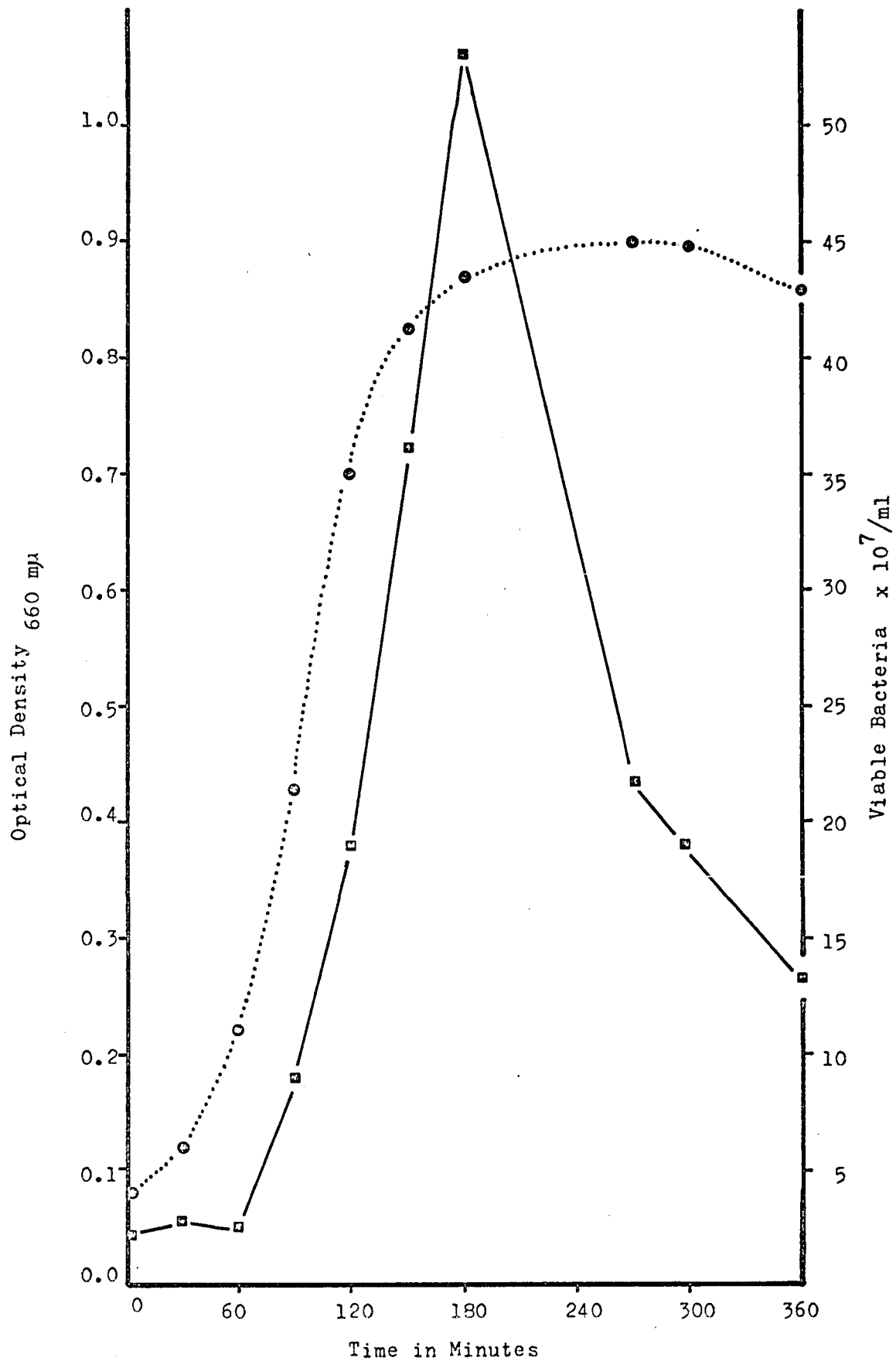


FIGURE 5: Viable count and optical density growth curves of strain 13.

## Propagation of the Bacteriophage

### Fluid Medium

A one per cent inoculum from an overnight culture was added to broth, and incubation at  $37^{\circ}$  C for one hour was allowed. Bacteriophage was then added at a multiplicity of infection (m.o.i.) of 0.1 or less and incubation was continued for  $2\frac{1}{2}$  hours. A higher m.o.i. was difficult to obtain because of the relatively low titers of the lysates used as a starter inocula. To decrease the number of bacteria would only serve to limit the ultimate yield of phage. A high m.o.i. might also tend to encourage lysogeny of the indicator strain.

After incubation, the bacteria were sedimented by centrifugation and the supernatant fluid was filtered under positive pressure through a Millipore GS 0.22  $\mu$  membrane filter. Although no loss of titer was observed upon filtration, the titers obtained never exceeded  $1-4 \times 10^8$  PFU/ml.

In order to be sure that the maximum titer to be expected under these conditions was being obtained, samples from such a propagation system were taken up to six hours after infection and assayed for PFU in the usual manner. As shown in Table III, a 2-3 hour period should not be exceeded in propagating this phage. The decrease in phage titer appears to occur at the same time the bacterial viable count has reached its peak.

TABLE IIIIncubation Time and Maximum Phage Yield

Incubation (Hours)	2	3	4	5	6
Titer x 10 <sup>7</sup> (PFU/ml)	17	16	13	11	9

Solid Medium

Confluent lysis of bacteria was obtained by spreading an appropriate amount of phage over the surface of an inoculated agar plate and incubating overnight. Semi-solid agar preparations were also prepared for confluent lysis. Some of the lysed plate preparations were frozen for 24-48 hours after incubation, while others were not; thawing of frozen plates was brought about at 37° C for 1-2 hours.

The phage was harvested from the non-frozen plates by adding 1 ml of phosphate buffer (Appendix I) to the surface and scraping the area with a glass spreader. When frozen plates were thawed, the expressed fluid was collected with a Pasteur pipette. All collected fluids were centrifuged and the supernatant fluids were assayed. The results of these experiments are shown in Table IV.

The frozen semi-solid plate method gives the highest phage titer, although the titer is not higher than that attainable

in broth. Since only 5 ml of fluid is expressed from these plates, this method of phage propagation is time consuming and inefficient for this phage.

TABLE IV

Production of Phage on Agar Plates

Type of Lysis	Frozen Plates	Non-frozen Plates
Surface lysis	$2.4 \times 10^7$ PFU	$1.4 \times 10^6$ PFU
Semi-solid lysis	$2.4 \times 10^8$ PFU	$2.3 \times 10^7$ PFU

## Proof of Lysogenicity

### Maintenance of the Lysogenic State

Eight colonies of strain 9 were picked individually and at random from a 24-hour sheep blood agar plate. Each colony was subcultured to blood agar plates and, after 24 hours of incubation, one colony was picked from each of these eight subcultures and the above procedure was repeated. In this way eight subcultures were passed a total of eight times. This random choice of colony selection over a number of passes was designed to free the culture of any extracellular "free" phage and to see if such colonies maintained the ability to release phage.

After the eighth pass on blood agar, a representative colony was picked from each of the eight subcultures and inoculated into Brewer's cooked meat medium for overnight incubation. These cultures were again passed in this same medium and treated as described below.

To eight 10 ml amounts of broth was added 0.4 ml of the overnight cultures respectively. After incubation for three hours, they were centrifuged and the supernatant fluid was assayed with the indicator strain 13 by the semi-solid agar overlay method.

It was found that 0.1 ml amounts of the supernatant fluids contained sufficient PFU to produce semi-confluent lysis.

When examined with a hand lens the plaques possessed turbid centers, suggesting lysogenization of the indicator strain. This experiment shows that each bacterium (as represented by a colony) sampled at random after a number of passes bears the potential of releasing bacteriophage.

#### Immunity of the Lysogenic Strain

A sample of semi-purified phage in phosphate buffer (see Purification and Concentration of Lysates) and having a titer of  $1.7 \times 10^7$  PFU/ml was diluted 10, 100 and 1000 times in broth and 0.1 ml amounts of these dilutions were plated with a three hour culture of one of the subcultured lysogenic colonies described above. Control plates using the indicator strain 13 assured the activity of the phage sample.

No plaques were formed on strain 9 whereas the control strain 13 demonstrated an uncountable number of plaques at the 1,000-fold dilution. This evidence shows that the lysogenic strain is resistant or "immune" to its own phage.

#### Inducibility of the Lysogenic Strain

Strain 9 was found to be inducible with u.v. light. The details of the induction experiments are found in the chapter entitled "Induction Experiments".

Lysogenization of the Indicator Strain 13

It was often noted that when direct plating of a relatively high titer lysate with the indicator strain was performed, the indicator strain showed no evidence of plaque formation or lysis, but rather demonstrated a confluent lawn of bacterial growth. Higher dilutions of the phage produced confluent lysis and individual plaques. This confluent lawn of bacterial growth does not suggest resistant growth as such, since one would expect to find resistant colonies appearing on plates with confluent lysis if such bacteria represented resistant members amongst the population as a whole, but rather lysogeny of the indicator strain at higher multiplicities of infection.

Some growth from such a plate was picked with a wire loop and spread on a blood agar plate. After a second pass on blood agar, two passes were made in cooked meat medium. When this strain was then used as an indicator with a known active phage preparation, no plaques were produced; i.e. it was immune. This immune strain was grown in broth for three hours, after which the culture was centrifuged and the supernatant fluid assayed for spontaneously released phage, as in the lysogenic strain, using the sensitive strain 13.

Only four plaques were observed on plating 0.1 ml of the supernatant broth. These were considered to be contaminating

phage carried over through the subcultures, since there were so few. An induction experiment was then performed on a two hour culture in the manner described in "Induction Experiments". An irradiation period of 25 seconds was used and 0.1 ml samples of irradiated and non-irradiated cells were inoculated into 9.9 ml amounts of broth respectively. After three hours of incubation it was observed that the control tube had developed considerable turbidity, while the irradiated sample had been greatly inhibited. Both cultures were centrifuged and the supernatant broths were assayed for phage.

No plaques were produced with either preparation when plated with the sensitive indicator strain. Since such a strain showed immune properties and characteristics of a lysogenic strain when irradiated, it might be proposed that a state of defective lysogeny has been established in the indicator strain and that mature phage particles cannot be produced here.



## Comparison of the Lysogetic and Indicator Strains

### Biochemical Comparison

Hiss serum sugars and litmus milk were inoculated with a few drops of the respective cultures as well as were iron acetate agar, sheep blood agar, human blood agar and egg yolk agar plates. The results of tests are reported in Table V.

The lysogenic strain 9 was the only Cl. perfringens strain studied in the experimental work which was non-haemolytic on sheep blood agar. When such plates containing non-haemolytic colonies were refrigerated overnight, a zone of Beta-haemolysis appeared around the colonies. Lecithinase (alpha toxin) has been reported as a "hot-cold" lysin active on sheep red blood cells (Mackie and McCartney, 1960) and since this strain produces lecithinase, this effect is to be expected; however, the absence of a haemolysin active on sheep blood cells in this strain is of interest.

### Animal Tests for Toxicity

Three strains of Cl. perfringens were used in the inoculation of guinea pigs to determine whether the in vivo response to these strains differed. One ml amounts of 48 hour cooked meat cultures of strains 1, 9 and 13 were injected into three guinea pigs re-

TABLE VBiochemical and Cultural Activities of Strain 9 and 13

Substrate	Strain 9	Strain 13
Litmus milk	clot, ag	clot, ag
Glucose	ag	ag
Maltose	ag	ag
Lactose	ag	ag
Salicin	-	-
Sucrose	ag	ag
Iron acetate ( $H_2S$ )	+	-
Egg yolk agar (1)	+	+
Sheep blood agar (2)	-	+
Human blood agar (2)	+	+

ag : acid and gas production

(1): lecithinase activity

(2): haemolysis

The preparation of egg yolk agar and sheep and human blood agars is described in Appendix I.

spectively. Gauge-16 needles were used for this purpose and in so doing were rotated and probed into the muscle tissue in order to establish more favourable conditions for the growth and toxin production of the organisms. The animals were observed for 48 hours and at this time were sacrificed. Strains 1 and 13 were known toxigenic strains.

Visual observation of the animals revealed apparent edema of the inoculated limb at both 24 and 48 hours. Autopsy demonstrated the following: marked necrosis, edema and lesions of the muscle tissue with a blood exudate in the peritoneal cavity noted with strain 1 and 13 infection; no obvious muscle damage but slight edema and necrosis with strain 9.

Strain 9 thus appears to be less toxic or non-toxic for the guinea pig. Further evidence is provided below. Three guinea pigs of equal size were inoculated as before with 1 ml each of a 48-hour culture of strain 9. One guinea pig was inoculated with 1 ml of a 48-hour culture of strain 1. The three animals inoculated with strain 9 showed edema at first, which later subsided. Autopsy on the fourth day revealed intact healthy muscle and no clinical sign of gangrene in these animals. The control animal inoculated with the toxic strain 1 produced typical gangrene.

Whether the missing haemolysin in strain 9 represents the toxic factor observed in the other strains is mere speculation.

Other Tests

Strain 13 was lysogenized by a high m.o.i. of phage in semi-solid agar. This strain, now resistant to the phage, was passed on blood agar and cooked meat. It was thought that if phage might be involved in the toxin production by way of repressing its formation, then non-haemolytic colonies might be found upon plating the now lysogenic indicator strain on sheep blood agar. No non-haemolytic colonies were ever observed in such experiments. Animal tests were not conducted with such a lysogenized strain.

Effect of Multiplicity of Infection (m.o.i.) on Phage Yield

It was often noted in experimental work that the development of a high titer lysate in broth culture was not possible. Experiments carried out in semi-solid agar layers on agar plates suggested that the indicator strain was being lysogenized at a relatively low m.o.i. Since an approximate  $1 \times 10^8$  bacteria in the soft agar seed layer could be lysogenized by  $1 \times 10^5$  PFU, it would seem that a m.o.i. of 0.001 might be sufficient to produce the lysogenic state in these bacteria. This is not to suggest that this low m.o.i. per se could establish lysogeny, but perhaps the number of phage released from the bacterial cells after the first lytic cycle would, upon a second cycle of infection, be large enough to influence this phenomenon, if indeed, m.o.i. has anything to do with lysogenization.

Semi-purified bacteriophage in phosphate buffer ( $1.7 \times 10^7$  PFU/ml) was diluted in serial 10-fold dilutions to a  $10^{-5}$  final dilution. Six tubes containing 8.9 ml of broth were inoculated with 0.1 ml of a three-hour culture of strain 13. These tubes were incubated for one hour, after which five of the tubes received one ml of a different phage dilution respectively. The sixth tube received one ml of broth and a viable count was performed on this sample by plating 0.1 ml samples of this culture on duplicate blood agar plates at an appropriate dilution.

After an incubation of  $3\frac{3}{4}$  hours the tubes were centrifuged and the supernatant broths were assayed for phage. The results are recorded in Table VI. The m.o.i. in this experiment is defined as the ratio of PFU to viable bacteria.

The data would imply that a m.o.i. as low as  $2 \times 10^{-4}$  provides the maximum lytic response in this system. Because the bacterial input is constant in each tube, it is not reasonable to propose that adsorption of phage to dead bacteria or debris plays a major role in the phenomenon. It is more likely that with a higher m.o.i. more cells can be multiply infected. The fact that the culture is not shaken during phage propagation might increase the possibility of multiple infection of uninfected bacteria surrounding a lysing bacterium.

TABLE VI

M.o.i. and Phage Yield

Description	Tube No.				
	1	2	3	4	5
Bacterial count/ml	$9 \times 10^6$ . . . . .				
Phage dilution	$10^{-1}$	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$
Phage input/ml	$1.7 \times 10^5$	$1.7 \times 10^4$	$1.7 \times 10^3$	$1.7 \times 10^2$	$1.7 \times 10^1$
Plating dilution	$10^{-2}$	$10^{-2}$	$10^{-2}$	$10^{-1}$	$10^{-1}$
PFU/plate	118	166	113	109	4
Phage output/ml	$1.2 \times 10^5$	$1.7 \times 10^5$	$1.1 \times 10^5$	$1.1 \times 10^4$	$4 \times 10^2$
M.o.i.	$2 \times 10^{-2}$	$2 \times 10^{-3}$	$2 \times 10^{-4}$	$2 \times 10^{-5}$	$2 \times 10^{-6}$
E.I. (1)	0	10	64.7	64.7	23.5

(1) The increment of phage is expressed as the number of times the total PFU/ml added as input has been increased by the end of the incubation period as described as output. For want of a better term, the expression E.I. (efficiency of increase) has been used.

### One-step Growth Curve

An inoculum of 0.6 ml from an overnight culture of strain 13 was added to 15 ml of broth and incubated for three hours. Then 0.2 ml of this culture was transferred to 17.8 ml of broth for an incubation period of one hour. After this time a viable count was performed on the culture by plating 0.1 ml samples of appropriate dilutions on blood agar plates.

A phage preparation in phosphate buffer containing  $1.5 \times 10^7$  PFU/ml was diluted 1:1000 in broth and two ml of this dilution was added to the 18 ml culture with very gentle mixing. As incubation continued, samples were taken frequently from this mixture and plated in 0.1 ml amounts either directly or after appropriate dilution in ice-cold broth. The semi-solid agar overlay method employing a three hour indicator culture was used for the plaque assay.

Three such experiments were done, but only one is presented which serves to characterize the burst. Fig. 6 depicts a typical growth curve of the phage. The points plotted on the graph represent the average of two plate counts.

There was a latent period of 45 minutes followed by a period of rapidly rising titer which reached its maximum 40 minutes later. The burst size, in terms of the whole culture,



was 17. In this particular experiment a second burst was shown to follow the first, commencing 100 minutes after the initial phage infection. Probably this would not be observable were it not for the long latent period enabling a stationary plateau to be reached before further multiplication occurred. The bacterial viable count in this experiment was  $4.3 \times 10^6$  bacteria/ml and the m.o.i. was 0.0008.

The other two experiments revealed identical latent and release periods although the actual burst sizes ranged from 65 to 300. There was a suggestion that this latter burst might be composed of two cycles of replication, the first of which would represent a burst of 30.

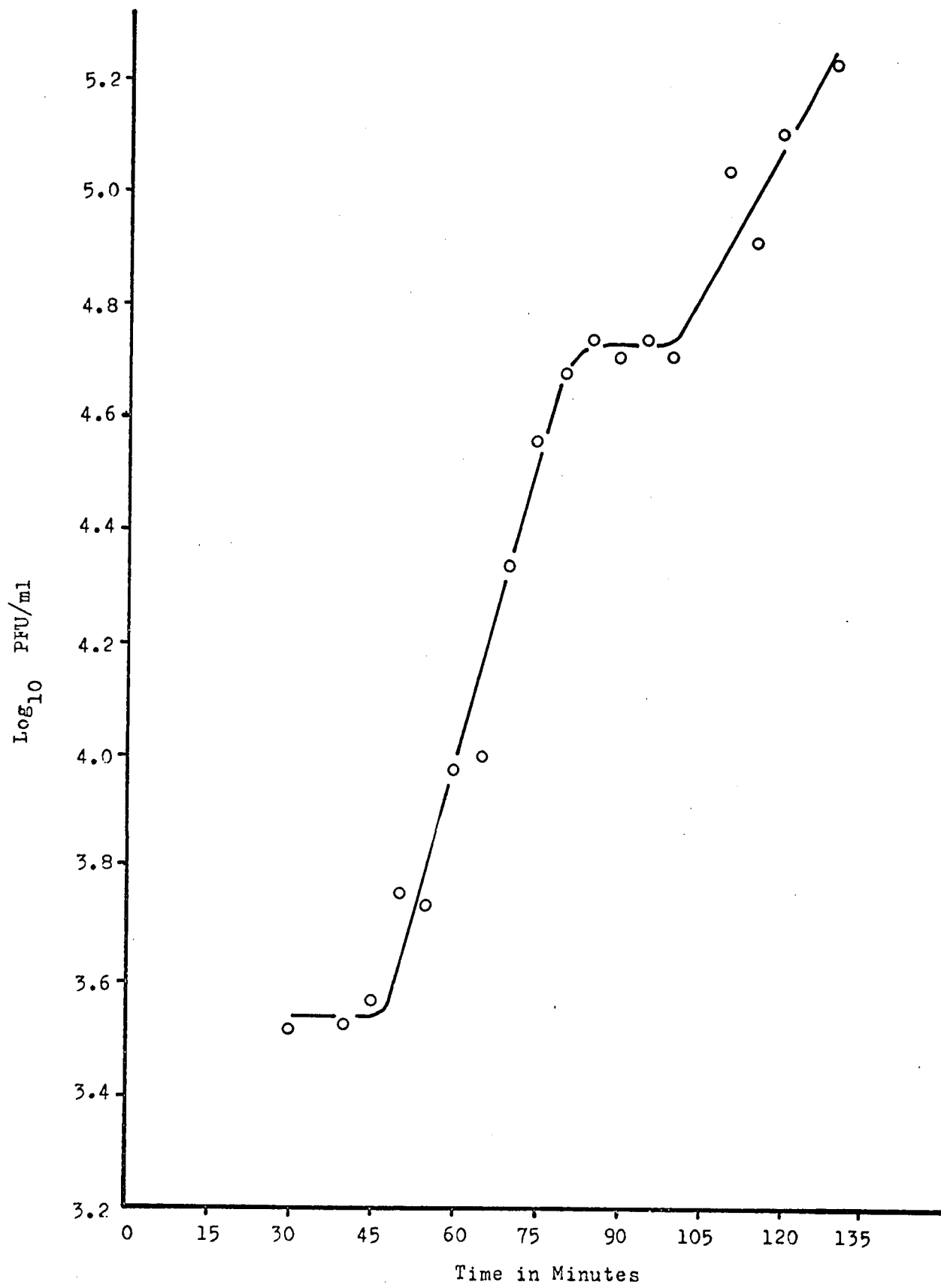


FIGURE 6: One-step growth experiment.

## Induction Experiments

### The Effect of U.V. Irradiation on the Lysogenic Strain

Strain 9 was grown in Brewer's medium overnight at 42°C and one ml of this culture was transferred to 15 ml of broth and incubated for eight hours. One ml of this eight hour culture was added to each of two 35 ml amounts of broth in centrifuge tubes, which were then incubated for 16 hours at 42°C. After incubation the bacterial cells were sedimented by centrifugation, the broth supernatant was discarded and the cells were washed twice in cold 0.85 per cent saline and resuspended in saline to the original volume. These two saline preparations were pooled to give 70 ml of a suspension of bacteria containing  $3.9 \times 10^7$  viable bacteria/ml.

Seven ml portions of this suspension were dispensed in Petri dishes and these were exposed to u.v. light for different durations of time ranging from one minute to eight minutes. A non-irradiated sample was used as a control. Suitable dilutions were made of the irradiated samples, and 0.1 ml amounts were spread on sheep blood agar plates with a wire spreader. The results appear in Table VII.

Although this experiment provided only an estimate of the bacterial sensitivity to u.v. irradiation, it suggested

that the greatest killing dosage was imparted within one minute, since 99.9 per cent of the population had been killed by this time. It seemed advisable, then, to expose the cells for less than one minute of irradiation in induction experiments.

TABLE VII

The Effect of U.V. Irradiation on the Lysogenic Strain

Time (Min)	Viable Count/ml $\times 10^2$	Per Cent Survival
0	390,000	100
1	440	0.11
2	130	0.033
3	2.5	0.00064
4	2.6	0.00067
5	2.8	0.00072
6	3.5	0.00090
7	1.2	0.00031
8	0.0	0.0

Induction of the Lysogenic Strain

A bacterial culture was prepared for irradiation as described in the previous experiment. Seven ml volumes of the

saline suspension of bacteria were placed in Petri dishes and exposed to u.v. light for various periods of time ranging from 20 to 120 seconds. Five ml portions of the irradiated samples were centrifuged and the sedimented cells were resuspended in five ml of broth. These cultures were incubated at 42° C for five hours, a non-irradiated control sample being treated similarly. After incubation the cultures were centrifuged and the supernatant broths were assayed for phage in the usual manner. The results appear in Table VIII.

TABLE VIII

U.V. Induction of Strain 9

<u>Irradiation Time (Sec)</u>	<u>Titer (PFU/ml)</u>
0	1.4x10 <sup>4</sup>
20	8.4x10 <sup>4</sup>
40	3.7x10 <sup>2</sup>
60	1.0x10 <sup>2</sup>
80	0.0
100	0.0
120	0.0

An irradiation period of 20 seconds caused a six-fold increment in phage titer, suggesting that strain 9 had been induced. Further experimental work justified this suggestion.

Attempts to Characterize an Induction Burst

Twenty ml of broth was inoculated with one ml of an overnight culture of strain 9. This culture was incubated for four hours, after which it was centrifuged to sediment the bacteria. The bacterial cells were washed twice with 0.85 per cent saline and resuspended in saline to the original volume.

Two Petri dishes containing seven ml each of this suspension were irradiated for 20 seconds and five ml of each irradiated sample were pooled. The bacteria were centrifuged and the sediment was resuspended in 10 ml of broth and distributed in one ml amounts into centrifuge tubes. Five tubes, so prepared, were incubated at 37° C. A control set of tubes containing one ml each of non-irradiated cells was put up in parallel. At the end of each hour of incubation one tube from each set was centrifuged and the supernatant fluid assayed for phage. The experiment ran for five hours.

This same experiment was repeated using a 2½ hour culture of the lysogenic strain for irradiation rather than a four hour culture. This experiment ran for seven hours.

Neither of these two experiments produced any results characteristic of induction. The titers of the test supernatant fluids often paralleled those of the controls and the few occasions

on which they exceeded the controls, the differences were not marked enough to establish that induction had occurred.

A third experiment, using a four hour culture of strain 9, differed from the first two experiments in that one ml of culture was removed from 20 ml amounts of irradiated preparations at one hour intervals. The one ml samples were centrifuged to sediment the bacterial cells and the supernatant fluid was removed for assay. It was thought that a 20 ml culture might develop anaerobic conditions more readily than the one ml samples previously used and that induction under more normal growth conditions might occur.

The results of this experiment appear in Fig. 7. Induction occurred in this attempt. A latent period of two hours was observed which was followed by a steady increase in phage titer. The induced culture titer was 22 times that of the control at a six hour sampling. The conditions of this experiment seemed to suggest that a larger volume of culture was necessary for this phenomenon, perhaps for the establishment of anaerobic conditions. It was thought that the yield might further be increased by diluting the irradiated bacteria in a larger volume of broth and, hence, not only improve anaerobic conditions, but also prevent excessive adsorption of released phage to bacteria and debris. Although the broth contained a

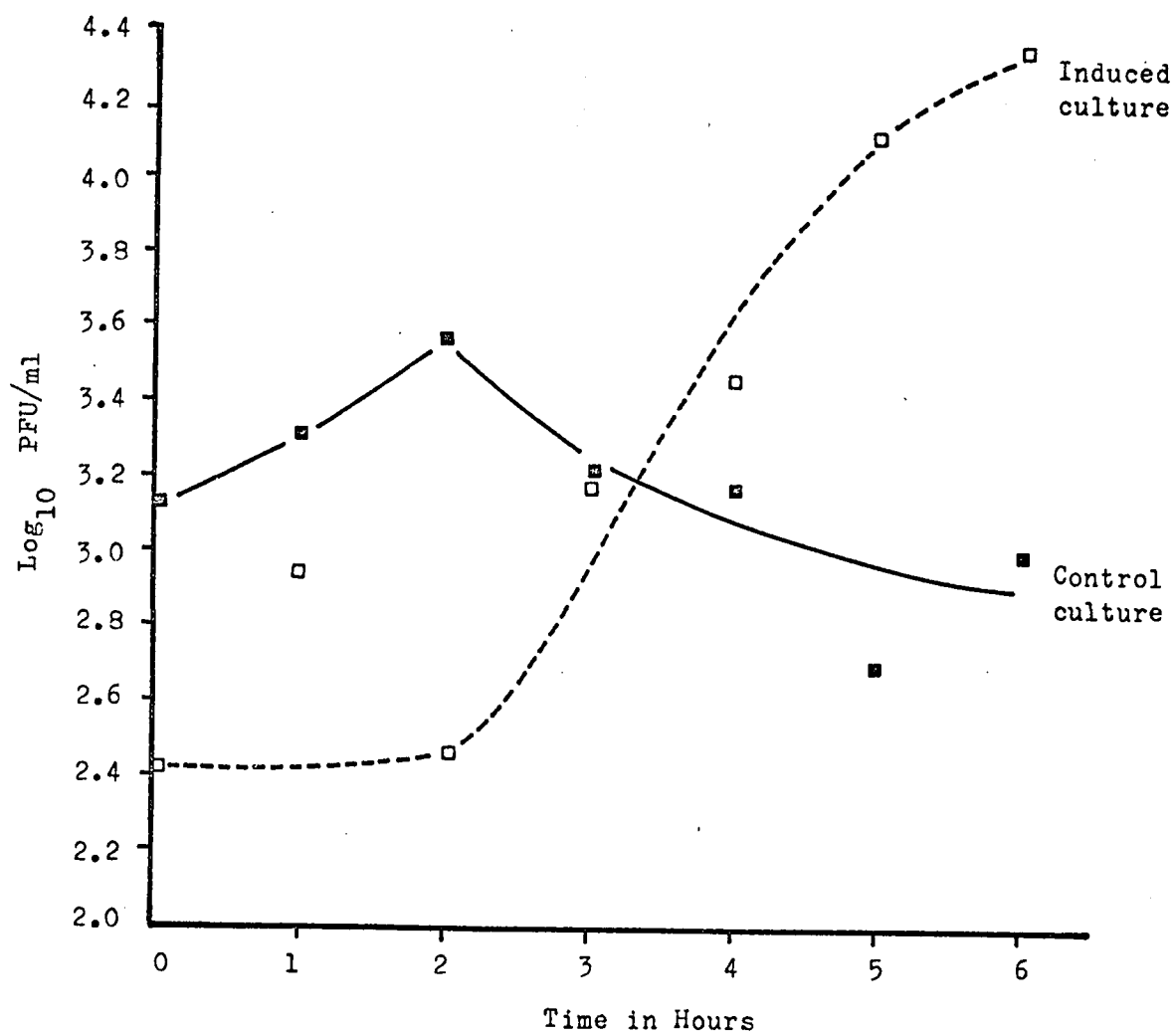


FIGURE 7: Characterization of induction with ultraviolet light.



reducing agent, excessive exposure to air would be detrimental to the growth of the organism.

#### Effect of Dilution upon Induction

A  $2\frac{1}{4}$  hour 20 ml culture of strain 9 was centrifuged, washed twice in 0.85 per cent saline and then resuspended in saline to the original volume. Seven ml of this culture was irradiated for 20 seconds. Immediately after irradiation a one ml sample of these cells was diluted in 100 ml of broth. One ml of non-irradiated culture was added to another 100 ml amount of broth as a control. After  $2\frac{1}{4}$  hours of incubation, 35 ml of each culture was centrifuged to sediment the bacteria, and the supernatant fluids were assayed for phage.

Induction in this experiment was very obvious. The control titer was  $9 \times 10^5$  PFU/ml and that of the induced culture was  $3.5 \times 10^7$  PFU/ml, an increase of  $3.4 \times 10^7$  PFU/ml. In this experiment it was noted that the turbidity of the u.v. treated sample lagged far behind that of the control culture. Microscopic observation of a sample from this irradiated culture revealed many lysed cells and those which were intact were of increased length and distorted morphology.

### One-step Burst Experiment with an Induced Culture

With the knowledge that a 100-fold dilution of irradiated cells was conducive for efficient induction, a one-step bursting experiment was again attempted.

A two hour culture of the lysogenic strain was prepared for irradiation as described above. Ten ml of the saline suspension was spread in a very thin layer over the bottom of a 15 cm Petri dish. After irradiating for 20 seconds, one ml of the suspension was added to 100 ml of broth at 37° C. As incubation progressed, two ml samples of the culture were taken at various intervals over a period of three hours and these were centrifuged to sediment the bacteria. The supernatant fluids were assayed at the completion of the experiment.

The induction curve obtained is presented in Fig. 8. Although a control curve was not plotted, the titer obtained at 180 minutes of incubation was determined by assay. The induced culture at this time possessed a titer 123.5 times that of the control. A definite burst occurred, starting between 60 and 75 minutes after irradiation and continuing for 90 minutes. The latent period appeared to be at least 15 minutes longer than that found in the one-step growth curve experiment and the release phase lasted 50 minutes longer. The increment in titer from zero time (30, 45 and 60 minute assay average) to the maximum release at 150 minutes is approximately  $1.1 \times 10^6$  PFU/ml.

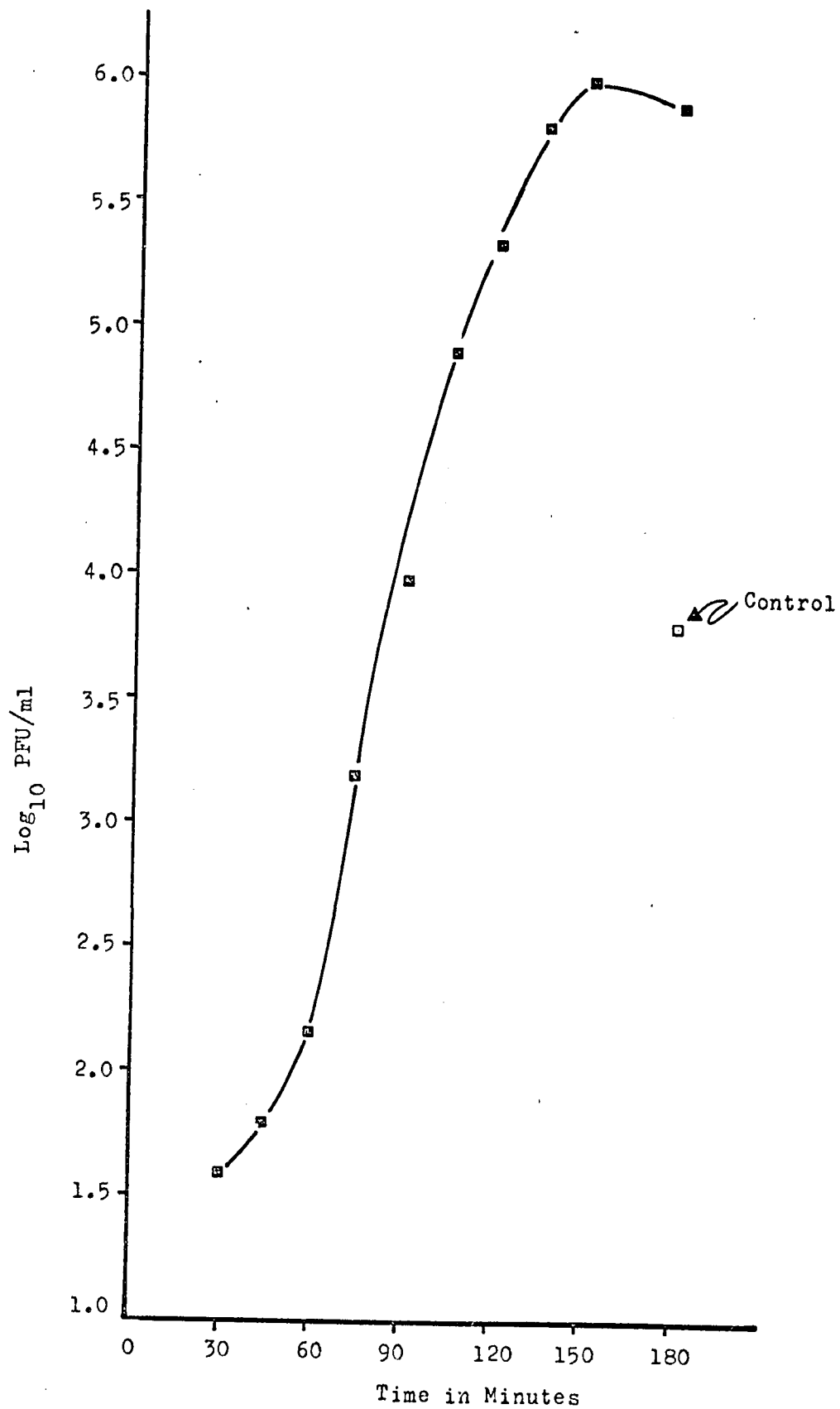


FIGURE 8: Induction burst in the lysogenic strain.

### The Optimum U.V. Dose for Induction

In the original experiment an exposure to u.v. light of 20 seconds was shown to produce the optimum induction of strain 9. The experiment, however, employed an 18-hour culture and dilution of the irradiated cells had not been made. The following experiment was designed to confirm the previous findings.

Five-ml amounts of washed bacteria from a two-hour culture were irradiated for various times and, after irradiation, 0.1 ml was taken from the sample and diluted in 9.9 ml of broth. A control tube containing non-irradiated cells was included. These cultures were incubated for 150 minutes, centrifuged and the supernatant fluids were assayed for phage.

The results are depicted in Fig. 9. Under the conditions of this experiment, the results were much more dramatic than those obtained in the earlier experiment. Sampling at 10 second intervals revealed that a higher peak could be obtained by using a culture irradiated for 30 seconds rather than 20, but the rapid fall in titer brought about by a further 10 seconds (total of 40 seconds) suggested that 30 seconds was very close to some critical point in either phage or bacterial sensitivity to u.v. light. Therefore, a 20-30 second exposure period appeared to be a safer level of irradiation for optimum induction. The maximum induction in this experiment yielded an increment of  $1.4 \times 10^6$  PFU/ml over the control titer.

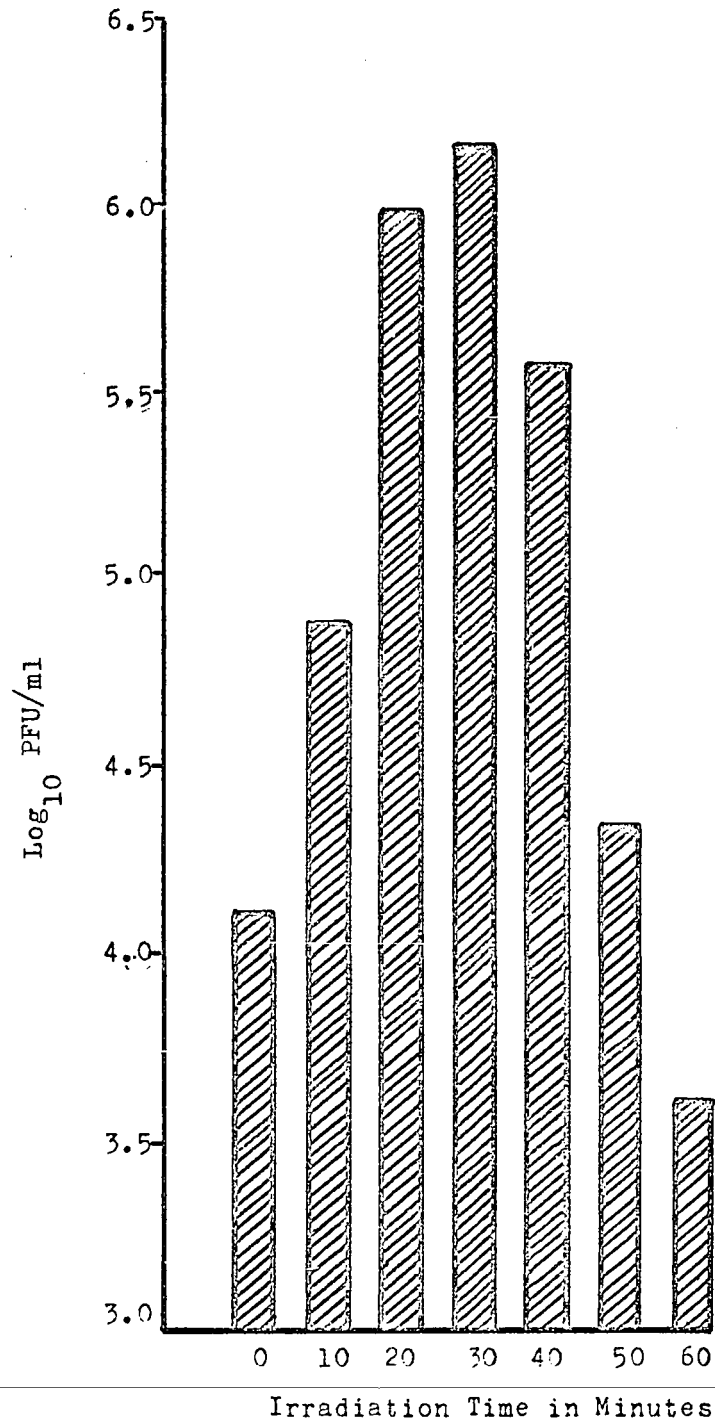


FIGURE 9: Induction of the lysogenic strain with ultraviolet light.

Induction as a Means of Producing High Titer Lysates

A two-hour culture was prepared for irradiation as in previous experiments. Ten ml amounts of this culture were irradiated for 20 seconds so that a total of 80 ml of irradiated cells was added to 500 ml of broth at 37° C. After 150 minutes of incubation the culture was centrifuged and the supernatant fluid was assayed for phage.

A titer of  $2.5 \times 10^7$  PFU/ml was obtained.

In an attempt to increase the yield, a smaller volume of broth was used as a diluent. About 70 ml of an irradiated culture was added to 20 ml of broth, and after 150 minutes of incubation, the culture was centrifuged and the supernatant fluid was assayed for phage.

A titer of  $7.1 \times 10^4$  PFU/ml was obtained. This result further emphasizes the need to highly dilute the induced bacteria if a reasonable yield of phage is desired. In terms of producing a high titer lysate, induction is not an attractive method, since the dilution necessary for a high burst to be realized counteracts the good yield of the burst.

The experiments described up to this point have demonstrated that titers exceeding  $2-4 \times 10^8$  PFU/ml were never attained in fluid or on solid media. Such titers were quite adequate for

a number of experiments but, for others, a much higher titer was essential and, of equal importance, was the purification of the lysate. For example, it was of interest to determine the type of nucleic acid carried by this phage; for such studies a purified preparation of high titer was required.

The following chapter describes the experimental procedures employed in the purification and concentration of both lysates.

## Purification and Concentration of Bacteriophage Lysates

### Centrifugation

Filtered phage lysates were centrifuged at four different speeds in the Sorval RC-2 refrigerated centrifuge and the Spinco Model L preparative ultracentrifuge. The sediments were resuspended in 1 ml of phosphate buffer containing magnesium sulphate and these, as well as the supernatant fluids, were assayed for phage.

The results are presented in Table IX. Only one set of conditions enabled concentration of the phage by centrifugation. Centrifugation in the Spinco centrifuge at 38,457 g caused a 7.4-fold concentration of the phage although there was a 70.9 per cent loss in total PFU during the process. Unfortunately, an assay of the supernatant fluid from the Sorval centrifugation was not made, but, from the titer of the resuspended sediment, it would appear that concentration had not occurred. The remaining two centrifugations suggest a loss in total PFU of at least 90 per cent and no concentration was evident.

It would seem, therefore, that this particular bacteriophage was very sensitive to high centrifugal forces. Probably the tail structure is damaged under stress.



TABLE IX

Purification and Concentration of Bacteriophage Lysates by Centrifugation

Sample	Gravity	Time (Min)	Input PFU/ml	Sediment PFU/ml	Supernatant PFU/ml	Total Input PFU	Total Recovery PFU	Per cent Recovery
10 ml	31,500	60	$2.7 \times 10^7$	$2.3 \times 10^7$	-	$2.7 \times 10^8$	-	-
35 ml	38,457	60	$1.0 \times 10^8$	$7.4 \times 10^8$	$8.1 \times 10^6$	$3.5 \times 10^9$	$1.0 \times 10^9$	29.1
13 ml	78,000	90	$2.7 \times 10^7$	$2.5 \times 10^7$	$2.8 \times 10^5$	$3.5 \times 10^8$	$2.9 \times 10^7$	8.2
13 ml	128,000	90	$2.7 \times 10^7$	$2.1 \times 10^7$	$1.2 \times 10^6$	$3.5 \times 10^8$	$3.6 \times 10^7$	10.3

Total input = volume x input/ml

Total recovery = sediment plus total supernatant

### Magnesium Pyrophosphate Gel

The method of Schito (1966) was tested as a means of purifying and concentrating bacteriophage lysates (See Appendix II). A 100 ml lysate was treated as described by Schito, and samples were removed at various stages of the procedure to check the efficiency of the method. About 30 ml of potassium phosphate buffer was used to elute the phage from the gel.

The results are presented in Table X. These data suggested an effective concentration of over three times.

TABLE X

#### Magnesium Pyrophosphate Gel Purification

Sample	Titer (PFU/ml)	Per Cent Recovery
Broth Lysate	$2.4 \times 10^7$	100.00
Dialyzed lysate	$1.7 \times 10^7$	70.83
Discarded broth supernatant	$3.4 \times 10^5$	1.42
Distilled water wash	$1.9 \times 10^5$	0.79
Buffer eluate (final product)	$7.8 \times 10^7$	325.00

In order to study the selectivity of the gel adsorption method, the supernatant fluid of a three hour bacterial culture

was processed in parallel with an uninoculated broth and a phage lysate in broth containing  $1 \times 10^8$  PFU/ml. After elution with potassium phosphate buffer (250 ml), the optical densities of the eluates were measured in a Zeiss PMQII spectrophotometer using wavelengths ranging from 230 to 300 m $\mu$ . The adsorption of ultraviolet light in this same range was also determined for an untreated sample of broth.

The absorption curves obtained are presented in Fig. 10. These observations suggested that some u.v. absorbing materials had been adsorbed by the gel; however, the u.v. spectrum obtained from the treated medium and culture did not resemble that of the phage preparation. The 260/280 absorption ratio was very low in the case of the medium. A 1:100 dilution of untreated broth had a much higher 260/280 ratio, indicating that the purification procedure had removed a quantity of material which absorbed heavily at 260 m $\mu$ , possibly nucleotides. The phage preparation showed a high 260/280 ratio of 1.55; however, later studies (see Nucleic Acid Studies) showed that bacterial nucleic acid can also be adsorbed by the gel, which could be contributing largely to the optical density reading at 260 m $\mu$ .

Although Schito suggested that his T phage preparation was pure after using Difco Brain Heart Infusion Broth as a medium, the present experiments indicated that contaminating substances from the medium can be adsorbed by the gel.

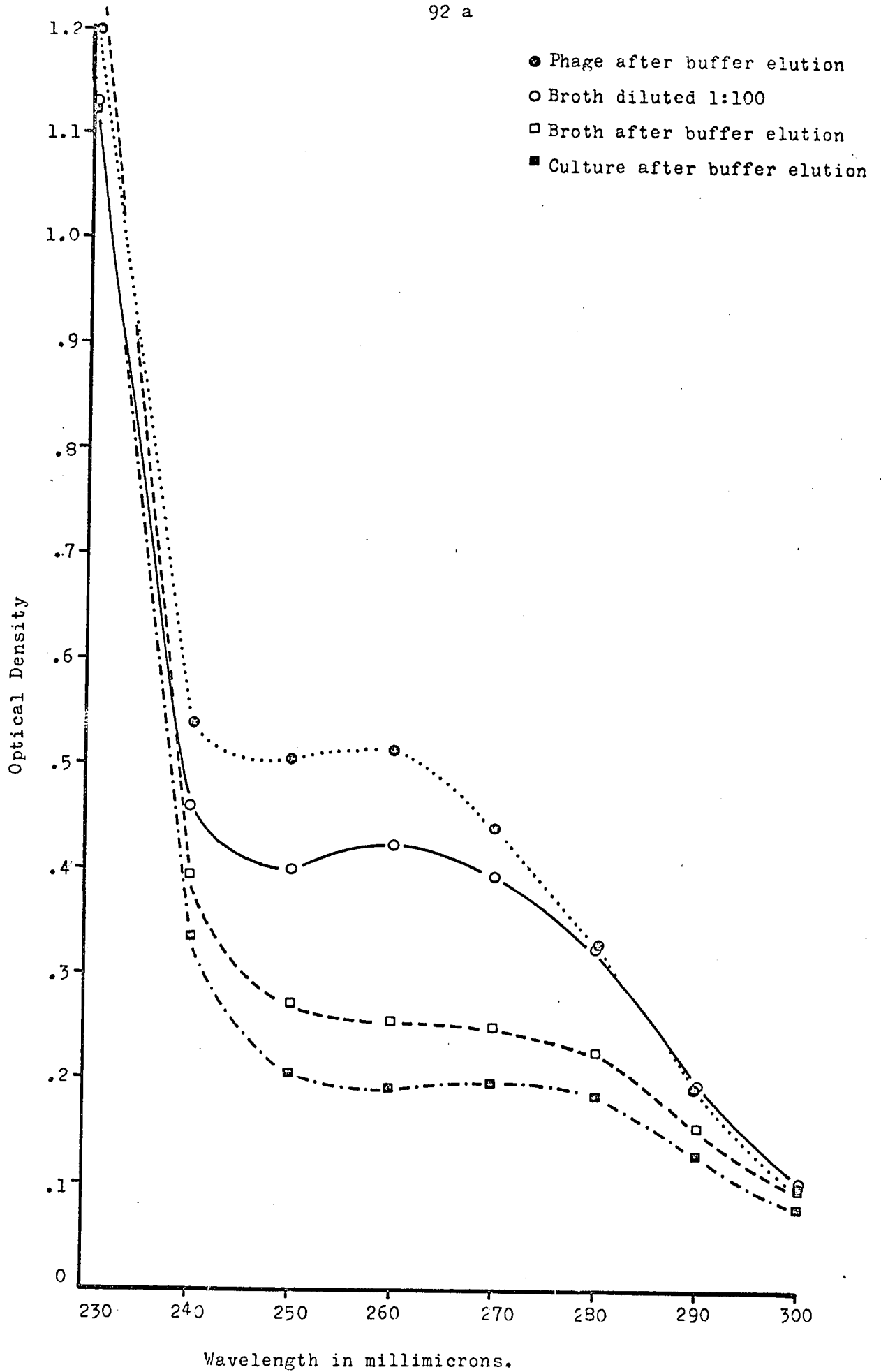


FIGURE 10: Spectrophotometric study of gel eluates.

### Flash Evaporation

Concentration of the bacteriophage in broth or buffer was very effective when the fluid was flash evaporated at  $37^{\circ}\text{C}$  under a pressure of 10 mm of mercury. A Buchler flask evaporator was employed for this purpose (Buchler Instruments, Fort Lee, New Jersey). The increase in phage titer was directly proportional to the decrease in fluid volume, although the broth samples became very viscous upon extreme concentration. Concentrations of 60-70 times could be approached in this manner.

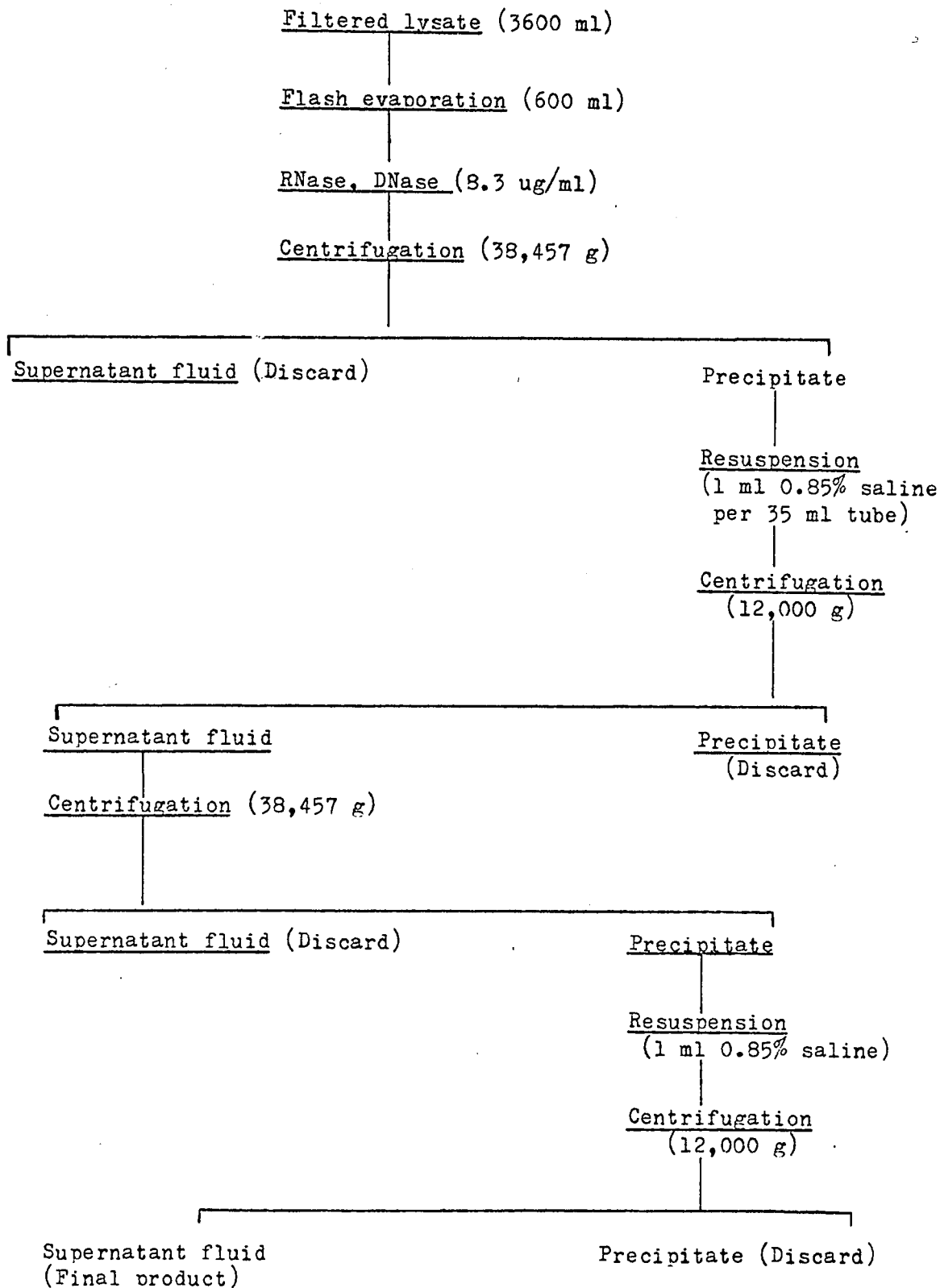
### Flash Evaporation and Differential Centrifugation

With the knowledge that a centrifugal force of 38,457 g could concentrate the phage up to seven times the original titer, a technique involving flash evaporation and centrifugation was employed. Adams (1959) described such a technique.

Six 500 ml quantities of broth were each inoculated with four ml of an overnight culture and incubated at  $37^{\circ}\text{C}$  for one hour. Then 100 ml of a phage lysate containing  $1 \times 10^8$  PFU/ml was added to each bottle and incubation continued for  $2\frac{1}{2}$  hours. These cultures were then centrifuged to remove the bacteria and the pooled supernatant fluids were filtered through a Millipore GS 0.22  $\mu$  membrane filter. The scheme outlined in Fig. 11 was followed at this point. Flash evaporation was performed in a

FIGURE 11

Scheme of Purification of Bacteriophage by Flash Evaporation  
and Differential Centrifugation



"Precision" flash evaporator with a four liter capacity evaporator bottle (Precision Scientific Company, Chicago, Illinois) under a pressure of 20-30 mm of mercury and at a temperature ranging from 28-40° C.

Ribonuclease and deoxyribonuclease (Worthington Biochemical Corporation, Freehold, New Jersey) were added in 5 mg amounts to the 600 ml flash evaporated lysate before the initial high speed centrifugation, providing a final concentration of 8.3 µg/ml of each enzyme. After thorough mixing, the lysate was incubated at 37° C for 30 minutes.

Centrifugation was carried out in a Spinco Model L preparative ultracentrifuge with a centrifugal force of 38,457 g (No. 30 rotor) for one hour. The supernatant broth was removed from the centrifuge tubes by means of a Pasteur pipette attached to a suction line. Slow speed centrifugations were done in a Sorval RC-2 refrigerated centrifuge with a centrifugal force of 12,000 g (SS34 head).

Phage assays were made at various stages of the procedure and these results are recorded in Table XI. Although the volume of the initial lysate was reduced 3600 times (to a final 1 ml volume), the titer was increased only 70 times. The final product should probably receive another cycle of differential centrifugation; however, after considering the small volume of the final product and the relatively low yield, another cycle was not performed.

TABLE XIAssay of Bacteriophage Titers Throughout Concentration Procedures

Sample	Titer (PFU/ml)	Concentration Over Previous Titer
Original phage lysate	$4.5 \times 10^8$	-
Flash evaporated lysate after nuclease treatment	$1.36 \times 10^9$	3 times (1)
Supernatant fluid after first high and low speed centrifugation	$6.4 \times 10^9$	4.7 times
Final product	$3.16 \times 10^{10}$	4.9 times

$$\text{Total concentration: } \frac{3.16 \times 10^{10}}{4.5 \times 10^8} = 70$$

(1) This concentration factor theoretically should be 6; however, excessive foaming in the evaporator occasionally occurred and the temperature within the evaporator bottle would climb to near critical levels for the phage. The temperature increase occurred only rarely and was solely dependent upon the efficiency of the vacuum system. Such variations in conditions might account for the inefficient concentration experienced in this experiment.



## Temperature Sensitivity of the Bacteriophage

### Survival and Heat Activation

The phage preparation used in these experiments was produced by centrifuging a broth lysate in a Sorvall RC-2 refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Connecticut) at 37,000 g for three hours and resuspending the "pellet" in 1/30 of the original volume of phosphate buffer containing  $2 \times 10^{-3}$  M-magnesium sulphate. The titer obtained was  $9 \times 10^5$  PFU/ml.

Two ml of this semi-purified phage was added to 18 ml of broth. After mixing well, this 1:10 dilution was added in two ml amounts to carefully cleaned glass test tubes maintained at a constant temperature in a water bath. An electric stirring apparatus was used to assure an even water temperature throughout the experiment. Each experiment involved seven tubes, the first being a control tube which was placed in crushed ice immediately. The other six samples were kept in the water bath and at one-half hour intervals one tube was removed and placed in the crushed ice until assayed for phage survival. All platings were made in duplicate.

Temperatures of 70, 60, 50 and 40° C were chosen as tests for heat sensitivity. At 70 and 60° C virtually all the phage was inactivated after 30-60 minutes exposure. At 50° C,

however, there was a notable increase in phage titer over that of the control after 30 minutes of exposure, after which the titer fell at a steady rate. The latter part of the curve probably represented normal heat inactivation.

At 40° C there was also a notable phage "activation", although the increment in titer was neither so great nor so rapid as that experienced at 50° C. The 40° C temperature, however, did not inactivate the phage after activation so that the final titer was considerably higher than that obtained at 50° C.

In order to confirm the above observations, the experiments involving the 50 and 40° C temperatures were repeated and a 30° C temperature experiment was also included. Sampling periods were much more frequent in these experiments.

The results are recorded in Fig. 12. The previous findings were confirmed. When the phage suspension was heated at 50° C, a peak of maximum activation was achieved within 5-15 minutes, after which inactivation proceeded at a steady rate. Heating at 40° C caused a rapid activation during the first 15 minutes followed by a slower rate until a maximum titer was obtained at 90 minutes. No inactivation occurred at this temperature. Exposing the phage to 30° C caused a slow and small increase in phage titer for 30 minutes, after which there was no activation or inactivation.

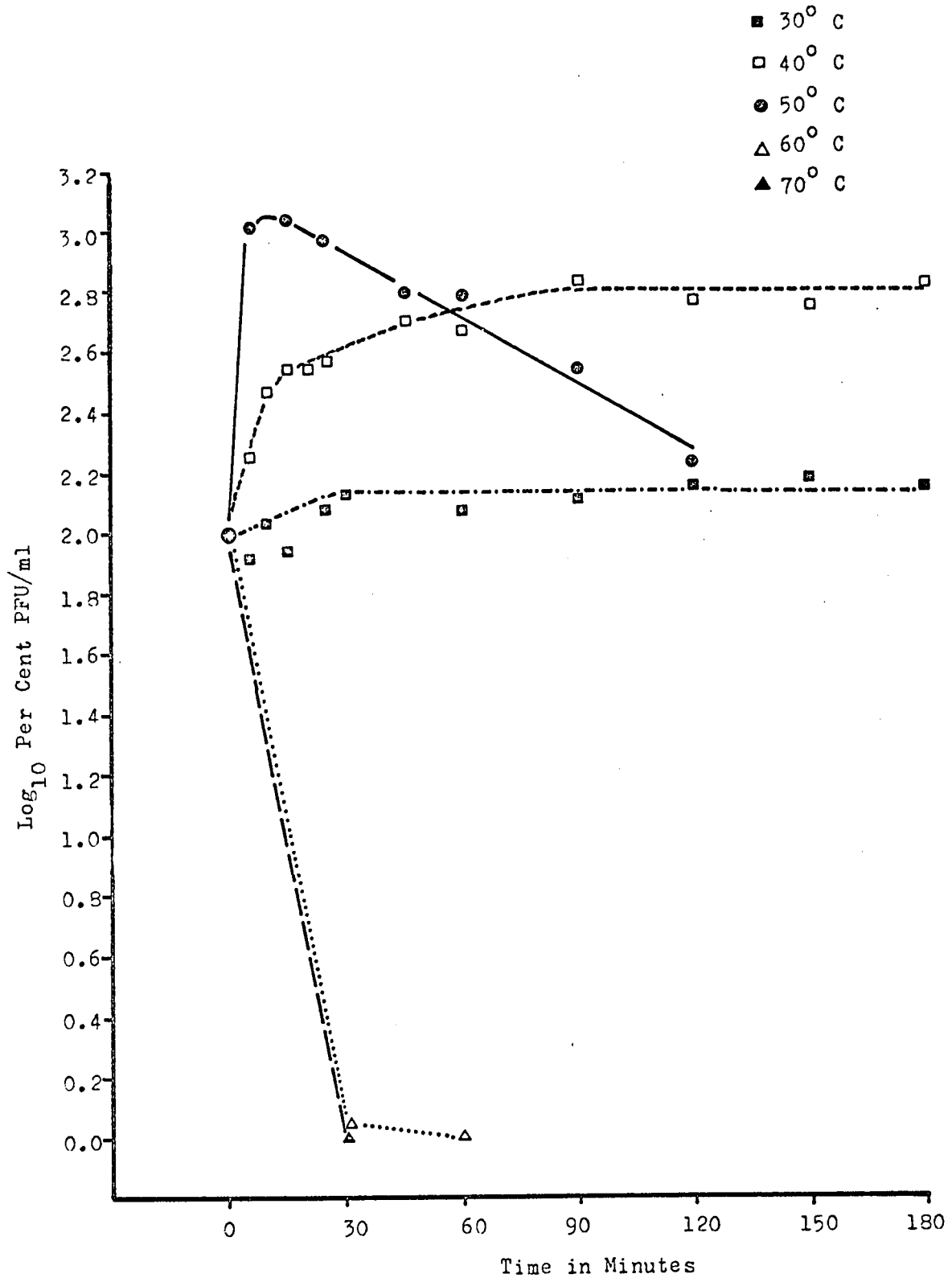


FIGURE 12: Temperature sensitivity of the Cl. perfringens phage.

Effect of Heat Activation on Phage Yield

A few experiments were performed in an attempt to explain the above phenomenon, one of which was to see if heating had in some way been able to influence the phage to produce a lytic response rather than a lysogenic response, where only part of the phage population might cause lysis.

A 40 ml culture of strain 13 was prepared by adding 1.6 ml of a three-hour culture to broth and incubating for 30 minutes. A viable count was then performed on this culture by plating 0.1 ml amounts of appropriate dilutions on duplicate blood agar plates. During the 30 minute period a sample of phage in phosphate buffer was heated for 15 minutes at 50° C and then cooled.

One ml of unheated phage and one ml of heated phage were added to two Spectronic 20 spectrophotometer tubes respectively. Seven ml of the 30 minute broth culture was then added to each tube as well as to a tube containing one ml of broth, which served as a control. A fourth tube containing eight ml of broth was used as a blank. The optical density for each tube was read at a wavelength of 660<sub>mμ</sub> (red filter, red phototube). The tubes were incubated at 37° C and optical density readings were made every 15 minutes over a 2½ hour period. At the end of the incubation period the cultures were centrifuged to sediment the bacteria and the supernatant fluids were assayed for phage..

The optical density readings are recorded in Fig. 13. There appears to be a stationary phase in the O.D. increments between 75 and 90 minutes of incubation. This time interval roughly corresponds with the period of maximum phage release in a one-step growth curve. Both the heated and unheated phage cultures follow this pattern. The heated phage culture O.D. is only slightly lower than that of the unheated phage culture. An analysis of the data obtained is presented in Table XII.

TABLE XII

Analysis of Heat Experiment Data

Description	Data
Bacterial viable count/ml	$1.9 \times 10^7$
Unheated phage input/ml culture (A)	$7.38 \times 10^3$
Heated phage input/ml culture (B)	$5.63 \times 10^4$
Unheated phage output/ml culture (C)	$6.6 \times 10^6$
Heated phage output/ml culture (D)	$4.8 \times 10^7$
. . . . .	
Increment in unheated phage after propagation with culture (C/A)	894-fold
M.o.i.	$3.9 \times 10^{-4}$
Increment in heated phage after propagation with culture (D/B)	853-fold
M.o.i.	$2.96 \times 10^{-3}$
Input ratios (B/A)	7.6
Output ratios (D/C)	7.3

- Culture control
- △ Culture plus unheated phage
- Culture plus heated phage

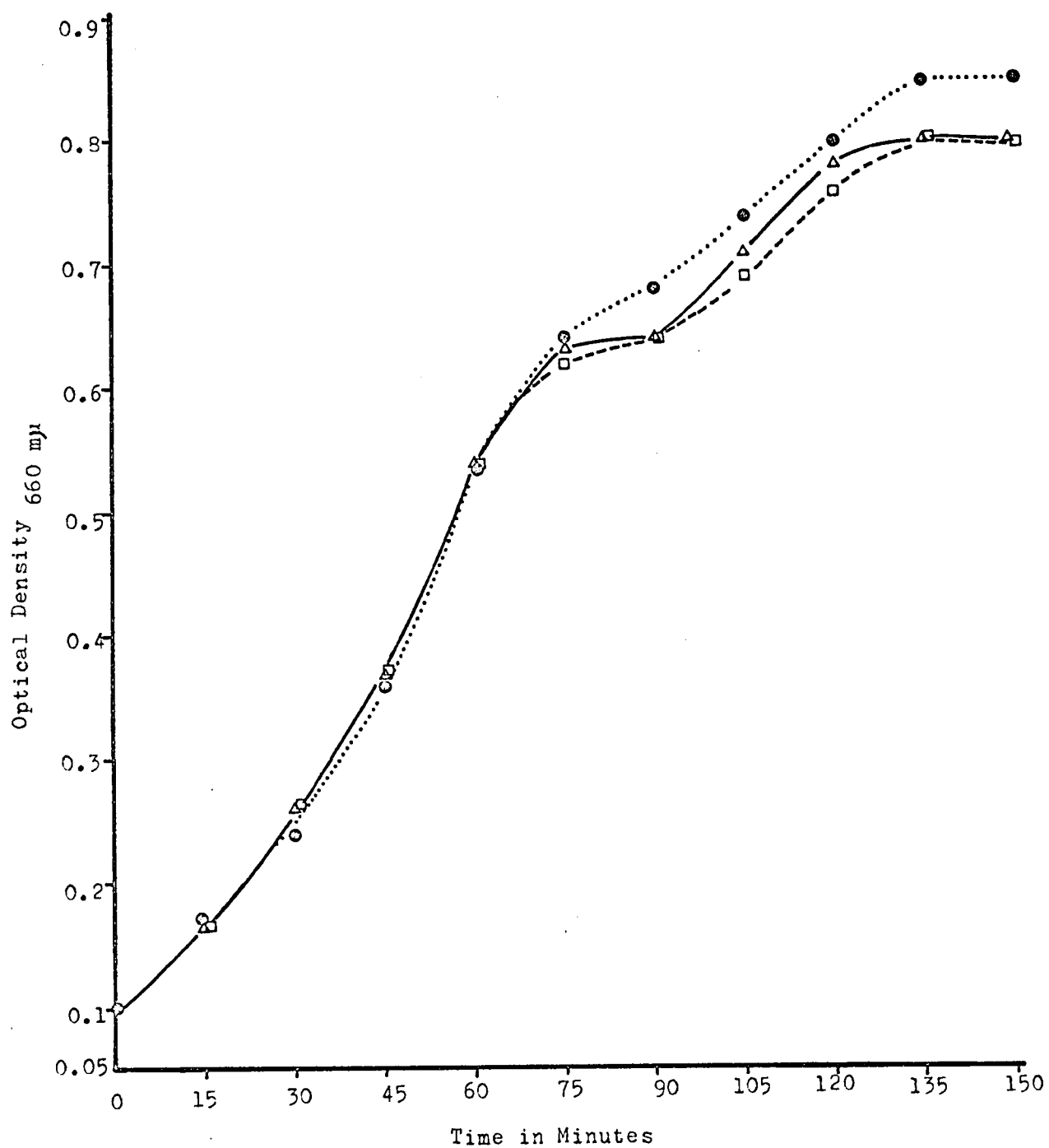


FIGURE 13: Effect of heat activation on bacteriophage production.

From these results it appeared that the heat treated phage did not undergo any change which might have led to a more lytic response. It seemed rather, that only the initial number of phages capable of infecting the bacteria had increased. The ratio of input titers paralleled the ratio of output titers. If the ability of the phage to produce a lytic response rather than a lysogenic response had been altered, one might expect to find a much larger increment in phage titer after propagation of the heated phage. The fact that the O.D. readings of the heated phage culture did not vary considerably from those of the unheated phage culture indicated that there was no marked increase in the lytic response.

It was apparent that the mode of phage preparation was a crucial factor in heat activation. Once the phosphate buffer preparation had been activated by heat, it could not be activated further by a second heating. The lysate obtained by a normal infectious cycle in broth (no purification) could never be activated by heating. This fact suggested that heat did not activate phage particles under normal conditions. Broth itself, cannot suppress activation because the phosphate buffer preparation was diluted ten-fold in broth for the original activation experiments. Three hypotheses are proposed to explain this phenomenon:

- (1) The phage particles have clumped or "agglutinated" in some

manner during the centrifugation procedure and heat serves to break up such aggregates. (2) The magnesium ions present in the buffer exert some influence either upon adsorption or some enzyme system. (3) The lysate containing phage after a normal infectious cycle contains some suppressing substance which prevents activation by heat and only after partial purification is this substance reduced to a level where it can be destroyed by heat.

#### High Speed Centrifugation and Heat Activation

In order to determine whether clumping might occur with high speed centrifugation, a broth lysate containing approximately  $1 \times 10^6$  PFU/ml was divided into two 30 ml portions and centrifuged in the Spinco preparative ultracentrifuge (Rotor 30) at 78,000 g for five hours. After centrifugation the supernatants were carefully removed by suction and the two "pellets" were resuspended in 5 ml of distilled water and broth respectively. These suspensions were diluted ten-fold in broth and two ml of each sample was heated for 15 minutes at  $50^{\circ}$  C, while the remainder served as the unheated control. After cooling the heated portions, a plaque assay was performed.

No activation occurred, but rather inactivation, upon heating. The degree of survival in each resuspension fluid was similar : water, 54.3 per cent; broth, 58.9 per cent. Since



this centrifugation period was much longer and more severe than that used in the preparation of the phage in phosphate buffer, it was felt that "clumping" would have occurred in the present experiment if such a phenomenon existed. It is interesting to note that this was one of the few experiments in which the phage was not inactivated by such a centrifugal force. It also appeared that the phosphate buffer containing magnesium was a requirement for phage activation.

#### Effect of Magnesium on Heat Sensitivity

Wallis, Smith and Melnick (1964) have reported the activation of Reovirus by heat in magnesium chloride solutions. The temperatures used and the curves plotted for the results very closely resemble those of the above experiments. Therefore, the following experiment was devised to determine whether magnesium plays a role in the activation of the clostridial phage by heat. The phosphate buffer in which this phage preparation was maintained contained  $2 \times 10^{-3}$  M-magnesium sulphate. The broth itself, probably contained some quantity of magnesium; such a quantity was not taken into consideration in the experiments which follow.

A 1 M-MgSO<sub>4</sub> solution was made in broth by dissolving 6.1625 g of MgSO<sub>4</sub> in a total volume of 25 ml broth. Serial 10-fold dilutions were made to a concentration of  $10^{-3}$  M-MgSO<sub>4</sub> by adding

0.5 ml of solution to 4.5 ml of broth. To each tube containing 4.5 ml of broth and a different  $\text{MgSO}_4$  concentration was added 0.5 ml of phage which had been prepared in broth and diluted in broth to contain about  $2-4 \times 10^4$  PFU/ml..

Two ml volumes of each preparation were placed in pre-warmed glass tubes in a water bath at  $50^\circ \text{C}$ . After an exposure of 15 minutes to this temperature, the tubes were cooled in an ice bath and the contents assayed for phage. Unheated control assays were also performed.

The results are recorded in Fig. 14. It would appear that magnesium played no particular role in heat activation but did play a role in increasing the phage titer when in higher concentration and in protecting phage from heat inactivation. A 1 M- $\text{MgSO}_4$  solution increased the PFU/ml 1.7 times and when heated there was a loss of 18 per cent of the titer, while there was a 41 per cent loss of titer when the broth control was heated. There is thus a definite protective effect against heat inactivation when magnesium ions are present. Even at  $10^{-2}$  and  $10^{-3}$  M concentrations there was a marked protective effect when compared to the control broth. It is proposed that the increase in PFU/ml in higher concentrations of magnesium is due to improved adsorption of the phage to the bacterium. The 1 M- $\text{MgSO}_4$  solution would become  $3.3 \times 10^{-2}$  M when diluted with the semi-solid agar and bacterial

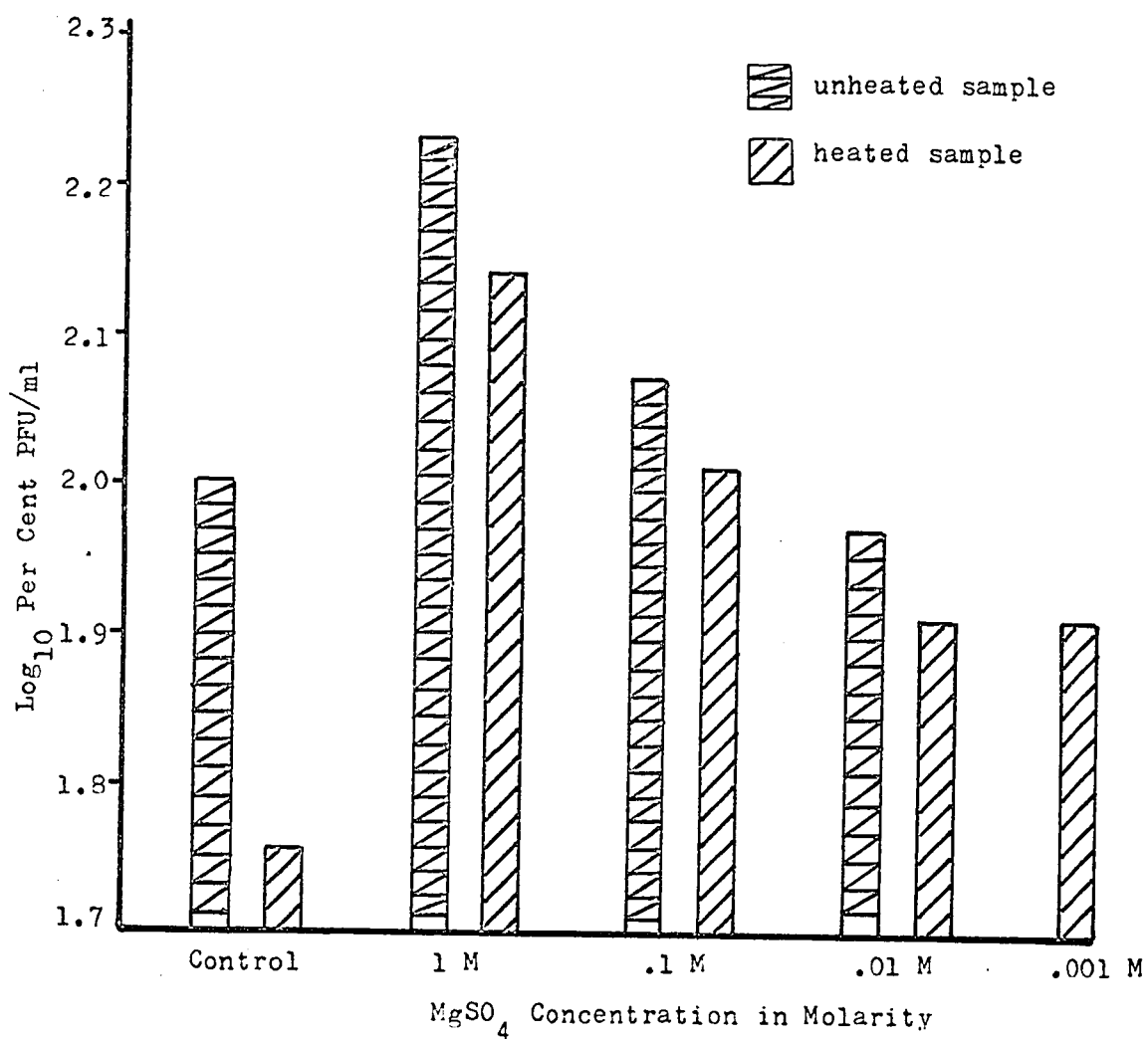


FIGURE 14: Effect of magnesium ion on heat activation.

inoculum on an agar plate. From the results of this experiment it was concluded that magnesium ions are not responsible for heat activation.

#### Effect of Dilution on Heat Activation

In an attempt to determine whether a suppressing substance was involved in the heat activation phenomenon, the following experiments were conducted.

A broth lysate diluted to contain about  $1 \times 10^4$  PFU/ml was further diluted in broth in 10-fold serial dilutions up to a  $10^{-4}$  dilution. Two ml of each dilution was placed in test tubes and, after heating at  $50^{\circ}$  C for 15 minutes, the preparations were cooled and assayed for phage. Unheated portions of each dilution were also assayed as 100 per cent survival controls.

Another experiment conducted in parallel employed phosphate buffer as diluent. A starting titer of about  $1 \times 10^4$  PFU/ml was prepared by diluting a broth lysate in phosphate buffer. Thus, in both these experiments there was an initial dilution of a broth lysate in the order of 100-fold before the 10-fold dilutions were made.

To 2.7 ml of the  $10^{-4}$  dilution of broth lysate (not expected to contain any detectable PFU) was added 0.3 ml of phage in phosphate buffer and this preparation was assayed before and

after heating at 50° C. As a control, 0.5 ml of this phage preparation was added to 4.5 ml of broth and samples of heated and unheated material were assayed. The purpose of this part of the experiment was to determine whether the lysate broth might suppress activation by heat of this preparation.

The results are recorded in Fig. 15. The titers were expressed as the average of two plate counts on each sample. Due to very low plaque counts with the  $10^{-2}$  and  $10^{-3}$  dilutions of the phage, the results were not considered reliable for inclusion in this figure. The  $10^{-4}$  dilution did not demonstrate any PFU, as was anticipated. No activation of the broth lysate was observed when diluted in either broth or buffer. However, there appeared to be a decrease in heat inactivation as the phage preparations were diluted. Again, the protective effect of magnesium can be observed as shown by the consistently higher titers in the phosphate buffer after heating. If the broth lysate contained some suppressor substance, it should be present in the  $10^{-4}$  dilution of the lysate; however, there was no inhibition of phage activation by heat when the phosphate buffer preparation of phage was added to this lysate.

In conclusion, these experiments have revealed a number of interesting features involving inactivation of phage by heat, but little light has been shed on the "activation" phenomenon.

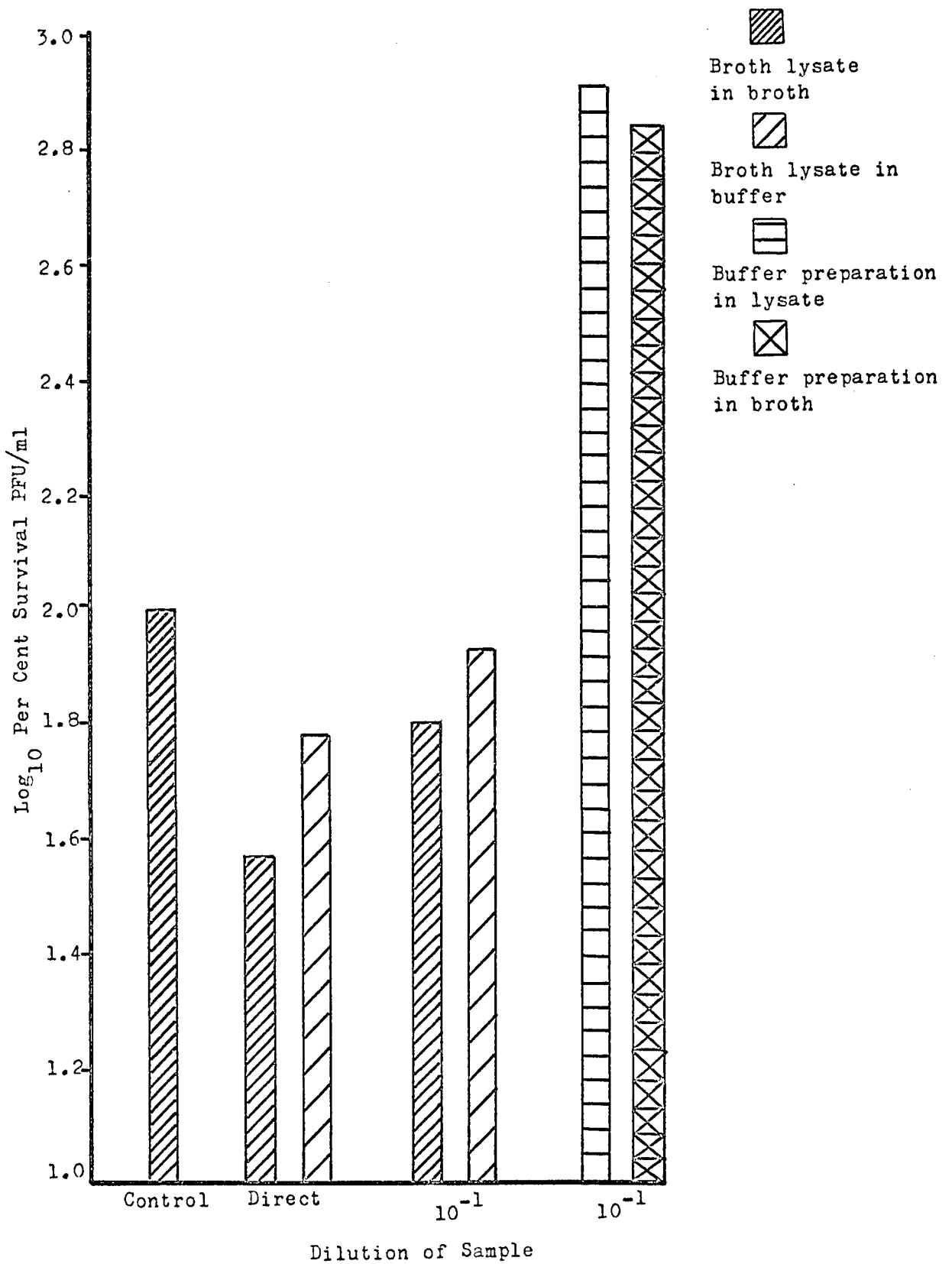


FIGURE 15: Effect of dilution on heat activation.

Sensitivity of the Bacteriophage to pH

The pH of broth was adjusted to various values ranging from three to nine by the addition of 1 and 10 N-HCl or 1 and 10 N-NaOH. The pH determinations were made with a Zeromatic Beckman pH meter (Beckman Instruments, Inc., Fullerton, California) at room temperature. Ten-fold dilutions of the phage in phosphate buffer containing about  $9 \times 10^5$  PFU/ml were made by adding 0.2 ml of phage to 1.8 ml of broth adjusted to the desired pH. After 30 minutes of incubation at room temperature, the preparations were diluted 10- and 100- fold in broth and assayed for surviving phage.

The results are recorded in Table XIII. A pH of 3 completely inactivated the phage and there was some inactivation at pH 4, whereas the titers at pH 5, 6 and 7 remained very stable. The titer was somewhat higher at pH 8 and 9, with pH 8 giving the highest titer. The titer obtained at pH 7 was considered a control value.

TABLE XIIISensitivity of the Bacteriophage to pH

pH	Titer (PFU/ml)
3	0
4	$1.04 \times 10^4$
5	$1.47 \times 10^4$
6	$1.25 \times 10^4$
7	$1.33 \times 10^4$
8	$2.31 \times 10^4$
9	$1.92 \times 10^4$



Sensitivity of the Bacteriophage to Ultraviolet Light

Three ml of the phosphate buffer phage preparation was diluted 10-fold in 27 ml of phosphate buffer. Five ml portions of this dilution were placed in each of five Petri dishes. Each sample was irradiated for a different number of seconds, ranging from 5 to 80. One non-irradiated sample served as a control.

The results are recorded in Fig. 16. Inactivation appears to follow a first order curve with about 99 per cent of the phage being inactivated after an exposure to u.v. light of 55 seconds.

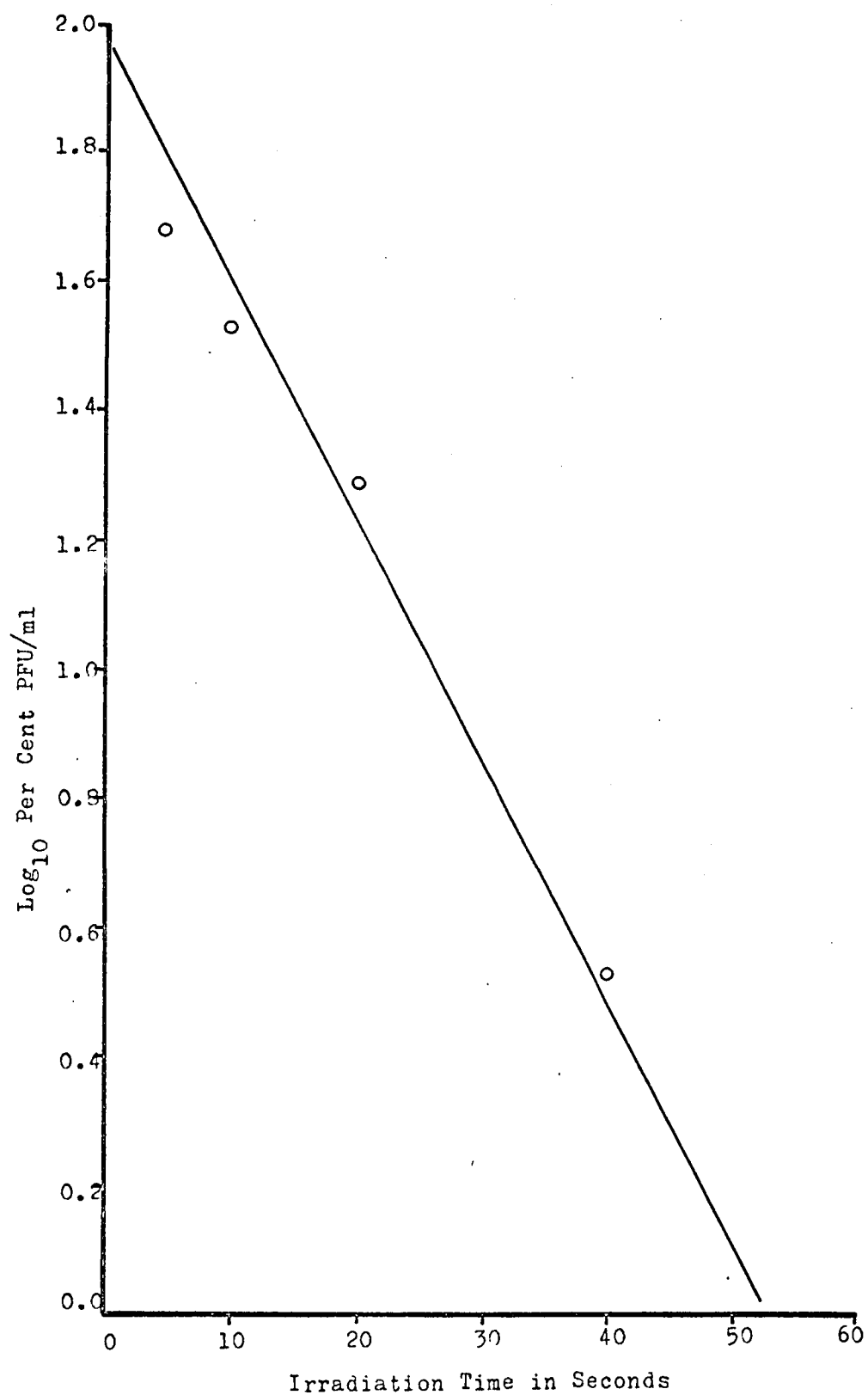


FIGURE 16: Inactivation of phage by ultraviolet light.

### Adsorption Experiments

Two ml of an overnight culture of strain 13 was added to 50 ml of broth and this culture was incubated for three hours. At this time a viable count was made on the culture by plating 0.1 ml amounts of appropriate dilutions on duplicate blood agar plates. One ml of a phage lysate containing about  $1 \times 10^7$  PFU/ml was then added to nine ml of the three hour culture. The lysate was assayed as the 100 per cent control sample.

The phage-bacteria mixture was incubated at  $37^{\circ}$  C in a water bath and, at various time intervals, 0.1 ml samples were removed and diluted in 9.9 ml of ice chilled broth. Five ml amounts of these dilutions were centrifuged to sediment the bacteria. The supernatant fluids were carefully removed and then assayed for phage which had not been adsorbed.

The above experiment was repeated with one modification; rather than take 0.1 ml samples from a 10 ml adsorption mixture, one ml portions of the mixture were placed in prewarmed glass tubes in a water bath at  $37^{\circ}$  C at the outset of the experiment and samples were taken at various times, but only one sample was taken from each tube. It was thought that this step might overcome any undue aeration and mixing of the mixture and hence provide a better estimate of adsorption, each tube remaining in a "resting" state until sampled.

The results are recorded in Fig. 17. There were no major differences observed between the results of the two experiments. There was a rapid adsorption of approximately 50 per cent of the phage within two to four minutes, after which adsorption continued at a decreased rate until 70-75 per cent of the particles were adsorbed by 20 minutes.

The bacterial viable count was  $4 \times 10^8$  bacteria/ml and the phage lysate contained  $9.4 \times 10^6$  PFU/ml. Since one ml of the phage preparation was added to nine ml of culture, the corrected assays should read  $3.6 \times 10^8$  bacteria/ml and  $9.4 \times 10^5$  PFU/ml. The m.o.i. was 0.0026.

Two other experiments employing lower numbers of bacteria and higher multiplicities of infection (m.o.i. = 1) failed to demonstrate any definite pattern of adsorption.

A number of experiments have been performed to characterize the temperate bacteriophage of C1. perfringens; however, its nucleic acid was still undetermined. In a very preliminary study of a partially purified phage lysate, a positive orcinol test was obtained when the preparation was extracted with hot perchloric acid, a factor suggesting the presence of RNA. It seemed very unlikely that a temperate bacteriophage would contain RNA or that a large phage with a tail structure would contain this nucleic acid, since all such phages of aerobic

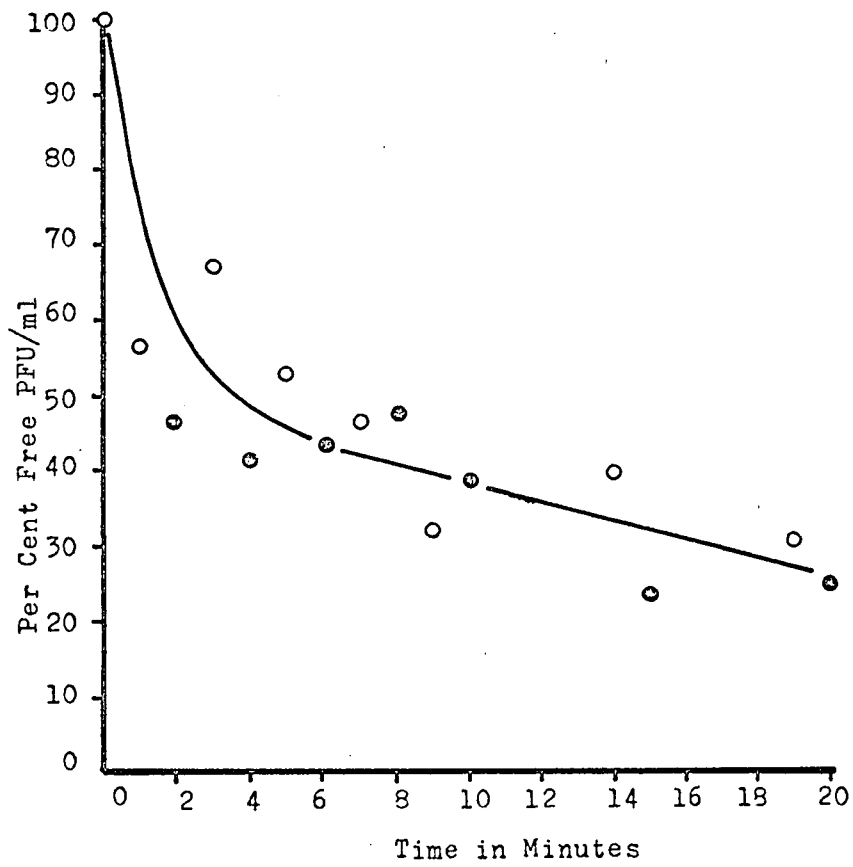


FIGURE 17: Adsorption of bacteriophage to strain 13.

○ Experiment 1  
● Experiment 2

bacteria have been shown to contain DNA; however, the nucleic acid of a Cl. perfringens phage had never been determined and so little was known about these phages, in general, that it was felt that a thorough study of the phage nucleic acid type was necessary. Such a study is recorded in the following chapter.

### Nucleic Acid Determination

The determination of the nucleic acid in this bacteriophage of Cl. perfringens was difficult because of the relatively low titers attainable in fluid medium and the chemical complexity of the medium itself. A number of purification and concentration techniques were employed in an attempt to overcome these difficulties.

### Chemical Determinations

The nucleic acid of the phage preparations was extracted by a modified Schneider technique and the extracted materials were examined for DNA and RNA by the diphenylamine and orcinol tests respectively (see Appendix II for experimental procedures).

### Magnesium Gel Preparation

Five hundred ml of a broth lysate containing  $3.7 \times 10^8$  PFU/ml was adsorbed by the magnesium pyrophosphate gel method of Schito and the eluted volume in potassium phosphate buffer contained a titer of  $2.6 \times 10^8$  PFU/ml. Ten ml of this preparation was treated with 0.77 ml of 70 per cent perchloric acid (PCA) to give an acid concentration of 5 per cent (v/v). To another 10 ml volume of phage was added 0.2 ml of a 500 µg/ml solution of ribonuclease providing a final concentration of enzyme of about 10 µg/ml. After incubation of this preparation at 37° C

for 30 minutes, a similar amount of deoxyribonuclease was added and incubation continued for another 30 minutes. This sample was then treated with PCA as in the first tube. It should be noted that the potassium of the buffer precipitated with PCA as potassium perchlorate; therefore, prior dialysis of the buffer preparation before extraction would be recommended.

A standard curve was prepared for various concentrations of yeast RNA, as tested with the orcinol reagents, and for various concentrations of sperm DNA, as tested with the diphenylamine reagents. Both nucleic acids were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. (See Fig. 18 for the standard curves.)

The hot PCA extract from the preparation not treated with enzyme was shown to contain 25  $\mu$ g of RNA as determined from the standard curve, but no DNA was found to be present. The nuclease treated material demonstrated no RNA. It would appear that the RNA observed in the non-enzyme treated sample was contaminating bacterial nucleic acid.

This experiment revealed two important facts: RNA could be adsorbed by the magnesium gel, and a total of  $2.9 \times 10^9$  PFU was an insufficient number of phages for nucleic acid determination.



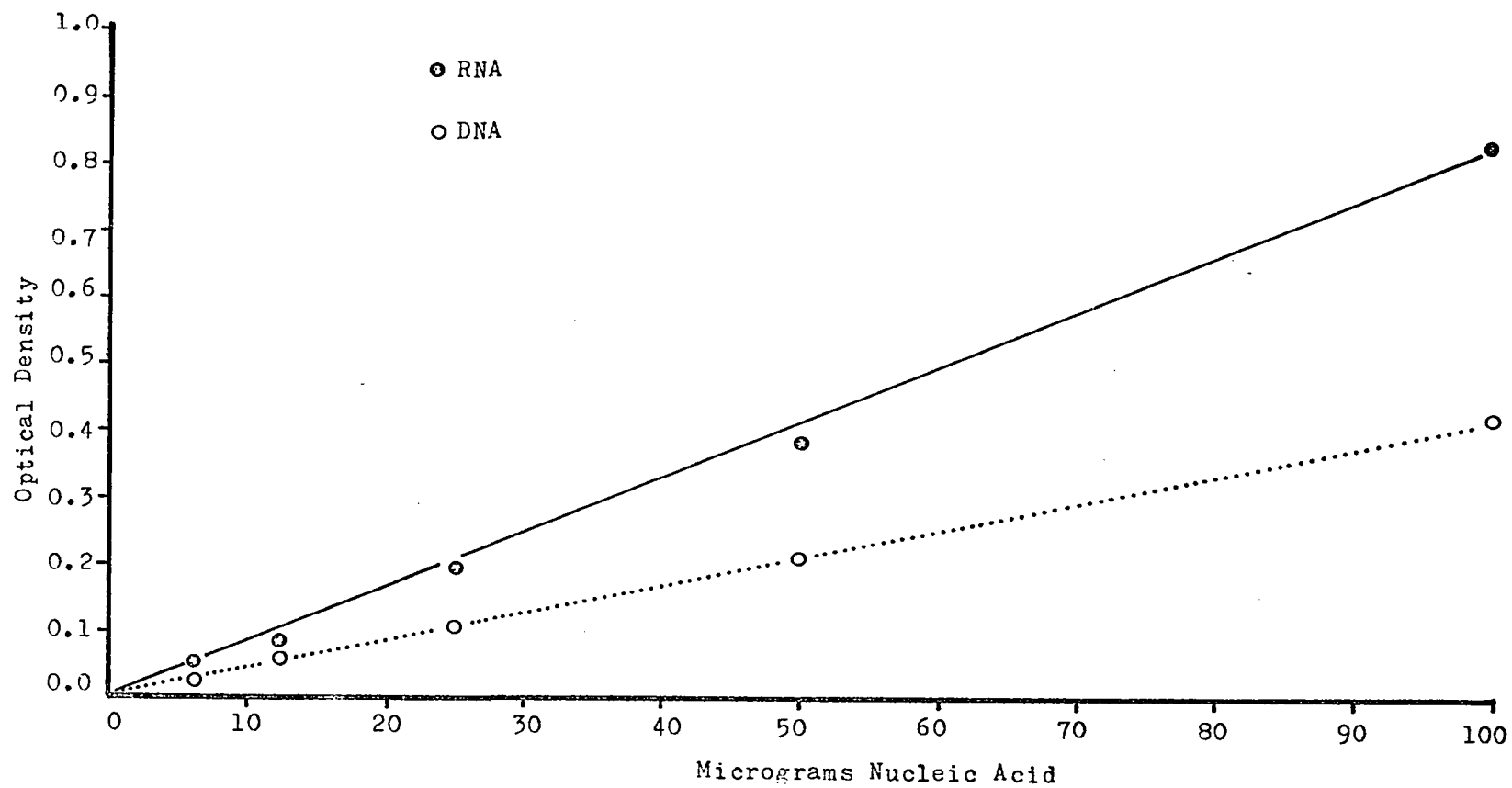


FIGURE 18: Standard curves for RNA and DNA as determined by the orcinol and diphenylamine reactions.

Flash Evaporated Preparation

Two liters of a broth lysate was treated according to Schito. The elution volume of phosphate buffer was 800 ml. This eluate was filtered through a Millipore GS 0.22  $\mu$  membrane filter and then dialyzed against two changes of distilled water for 48 hours at 4° C. This procedure was carried out in an Oxford dialyzing apparatus with a fluid capacity of eight liters. The failure of this dialyzed material to form a precipitate with PCA indicated that the high potassium content of the buffer had been greatly reduced or removed.

The phage preparation, now 900 ml in volume, was concentrated by flash evaporation at 38° C under a pressure of 10 mm of mercury to a volume of 10 ml, a concentration of 80-fold over the eluate volume. It was noted that soon after the air had been evacuated from the evaporation system, mucoid strands appeared in the fluid which tended to clump. This could not be due to concentration since it occurred very soon after the process had commenced. It was later shown that these strands were insoluble in water, ethanol and 1 N-HCl, but were soluble in 10 N-HCl.

The titer of the final product was  $1.46 \times 10^9$  PFU/ml. The material was brown and contained many clumps of fibrous substance. Eight ml of this preparation was treated with PCA and the nucleic acid determination was made.

The results are recorded in Table XIV. This experiment revealed the presence of both diphenylamine and orcinol reacting material in the hot PCA extract. The total number of PFU in the sample was approximately  $1.6 \times 10^{10}$ . The amount of DNA, corrected for the total phage (213  $\mu\text{g}$ ), almost equaled the amount of RNA (244  $\mu\text{g}$ ). The cold PCA extracts were also tested for RNA and DNA and it was shown that there was no diphenylamine reacting material in these samples, but there were extremely large amounts of orcinol reacting material present. It was, therefore, possible that some of this orcinol reacting material had not been removed from the final cold PCA extract of the test sample. Since it is very unlikely that this orcinol reacting material represents RNA, quantitative estimations of this material will be described as " $\mu\text{g}$  RNA equivalents", rather than " $\mu\text{g}$  RNA". Quantitative estimation of DNA will also be expressed in this manner until further evidence is provided that the material reacting with diphenylamine is the deoxyribose of DNA. Since this reaction is much more specific for DNA determinations than is the orcinol reaction for RNA, it is more likely that DNA is the nucleic acid involved in these studies.

The above procedure did not involve treatment of the test material with nucleases. Ribonuclease and deoxyribonuclease were, therefore, added to two ml of the test material to a final

TABLE XIVDetermination of Nucleic Acid in Flash Evaporated MaterialOrcinol Reaction

Sample	O.D. 665m $\mu$	$\mu$ g RNA Equivalents		
		$\mu$ g	Correction Factor For Dilutions	$\mu$ g/Total Phage
Cold PCA 1:5	.42	52.5	x5x2/3x8	1400
2nd wash 1:5	.75	93	x5x2/3x8	1550
Hot PCA	.98	122	x2/3x3	244
50 $\mu$ g RNA	.37			

Diphenylamine Reaction

Sample	O.D. 600m $\mu$	$\mu$ g DNA Equivalents		
		$\mu$ g	Correction Factor For Dilutions	$\mu$ g/Total Phage
Cold PCA	.078 (1)	0	x8	0
Hot PCA	.293	71	x3	213
50 $\mu$ g DNA	.216			

(1) This O.D. reading was due to non-specific absorbance. The test material possessed a slight brown colour. There was no peak of maximum absorbance at 600 m $\mu$ .

Reagent controls were incorporated in which 5 per cent PCA was substituted for the sample. Such controls were used as blanks.

concentration of 36  $\mu\text{g}/\text{ml}$  each. After incubation at  $37^{\circ}\text{C}$  for 30 minutes, the nucleic acid extraction was made. A total of 66  $\mu\text{g}$  RNA equivalents was present in the 2 ml sample. This was roughly 25 per cent of the value obtained in the above experiment and the volume tested was 25 per cent of the original volume. Such information suggested the presence of an orcinol reacting substance which was not soluble in cold PCA, was soluble in hot PCA and was ribonuclease resistant. Whether this substance was RNA or not could not be determined by this experiment. If it did represent RNA, it must have been protected from enzyme activity, suggesting that it could belong to the bacteriophage.

The diphenylamine reading was too low to be significant with this volume of test material. These results were somewhat confusing and it was concluded that a purer preparation of phage had to be studied.

#### Lyophilized Phage Preparation

Eighty-five ml of phage in potassium phosphate buffer containing  $2.6 \times 10^8$  PFU/ml was dialyzed against distilled water in order to remove potassium, after which the non-diffusable material was filtered through a Millipore GS 0.22 $\mu$  membrane filter. The fluid was then placed in a 500 ml Erlenmeyer flask and "shell frozen" by rotating the flask while holding it at an angle in

a solid carbon dioxide-ethanol bath. The frozen material was then lyophilized in a Virtis freeze drying apparatus (Virtis Company, Inc., Gardiner, New York) for 24 hours. The dried material was resuspended in 2.5 ml of distilled water, resulting in a 34-fold increment over the original volume.

One ml of this material was put in each of two test tubes. To one of these was added 0.5 ml of distilled water and to the other were added 125  $\mu$ g of ribonuclease and 125  $\mu$ g of deoxyribonuclease. After a total incubation of one hour at 37° C, the material was extracted with PCA and studied as described previously.

In the sample which was not treated with enzymes, 16  $\mu$ g of RNA was detected (Total RNA, 32  $\mu$ g). No RNA was detected in the enzyme treated sample and DNA was not detected in either sample. A considerable amount of orcinol reacting material was found in the cold extractions. This RNA would appear to be bacterial in origin. The number of PFU ( $8.8 \times 10^9$ ) was again too low for the detection of the phage nucleic acid.

#### Medium and Culture Control

This experiment was designed to show whether the medium or a supernatant fluid from a culture could contribute to the orcinol-reacting material observed in the previous experiments.

A  $3\frac{1}{2}$  hour 250 ml culture of Cl. perfringens was centrifuged to sediment the bacterial cells and the supernatant fluid obtained was used in this experiment. A parallel control was employed using 250 ml of broth. The two samples were purified according to the method of Schito. Approximately 120 ml of potassium phosphate buffer was used for the elution process. The eluates were then dialyzed against distilled water for 16 hours to remove potassium and finally flash evaporated to a volume of two ml, resulting in an approximate 60-fold concentration of the buffer eluates.

One ml of each preparation was then extracted and examined for orcinol-reacting material.

The orcinol test on these materials revealed no more than 6  $\mu\text{g}$  of RNA in the hot PCA extract. In fact, the O.D. readings may be too low to be significant (.038 and .054). The cold PCA extractions possessed orcinol-reacting material with values of 82.5  $\mu\text{g}$  RNA equivalents for the culture supernatant fluid and 120  $\mu\text{g}$  RNA equivalents for the broth. This experiment further demonstrated that this method of bacteriophage purification was not suitable when combined with flash evaporation, since there was too much undesirable material adsorbed by the gel which could be concentrated. It is very unlikely that the orcinol-reacting material is ribose, as such, since dialysis would have removed

such a low molecular weight substance if the gel adsorption had not already done so. Such material probably represents nucleotide or nucleoside complexes of the medium.

The "RNA" observed in the previous experiments would not appear to have any association with the medium or normal growth by-products of the organism and hence is characteristic of the phage infected culture.

#### Bacterial RNA and DNA

A rough estimate of the proportion of each nucleic acid was desired in order to determine how much bacterial RNA must be present in a lysate before bacterial DNA can be detected.

Strain 13 was grown for three hours in ten ml of broth, after which the culture was centrifuged and the resulting sediment was washed three times in 0.85 per cent saline. The washed sediment was resuspended in five per cent PCA at 4° C and the Schneider extraction was performed. An additional extraction was made with acetone in this experiment.

The tests revealed the presence of 742 µg RNA equivalents and 86 µg DNA equivalents in the total sample. There was 8.5 times as much "RNA" as "DNA".



### Differential Centrifugation Preparation

The bacteriophage used in this experiment was prepared by flash evaporation of bulk lysates, treatment with ribonuclease and deoxyribonuclease and subsequent differential centrifugation (see Purification and Concentration of Bacteriophage Lysates). Twenty-one liters of broth lysate were so processed to obtain approximately 14 ml of semi-purified bacteriophage with a titer around  $1 \times 10^{10}$  PFU/ml. This preparation was colourless, but had a slight opalescence.

Nine ml of the above preparation containing a total of  $8.3 \times 10^{10}$  PFU was extracted by the Schneider technique. The first supernatant fluid was labelled "Cold PCA I". The supernatant fluids from the two cold PCA washing were labelled "Cold PCA II" and "Cold PCA III" respectively. The precipitate obtained with cold PCA treatment consisted of a small blue-black pellet. Each supernatant fluid was studied with the Zeiss PMQII spectrophotometer employing 1 cm silica cuvettes, and the u.v. absorption spectra are presented in Fig. 19. The orcinol and diphenylamine tests were then performed on the Cold PCA III and Hot PCA extracts.

The results of the chemical tests appear in Table XV. From the u.v. absorption curves it appeared that most of the highly absorbing materials had been removed by the second washing. There was no particular absorption peculiar to the third PCA

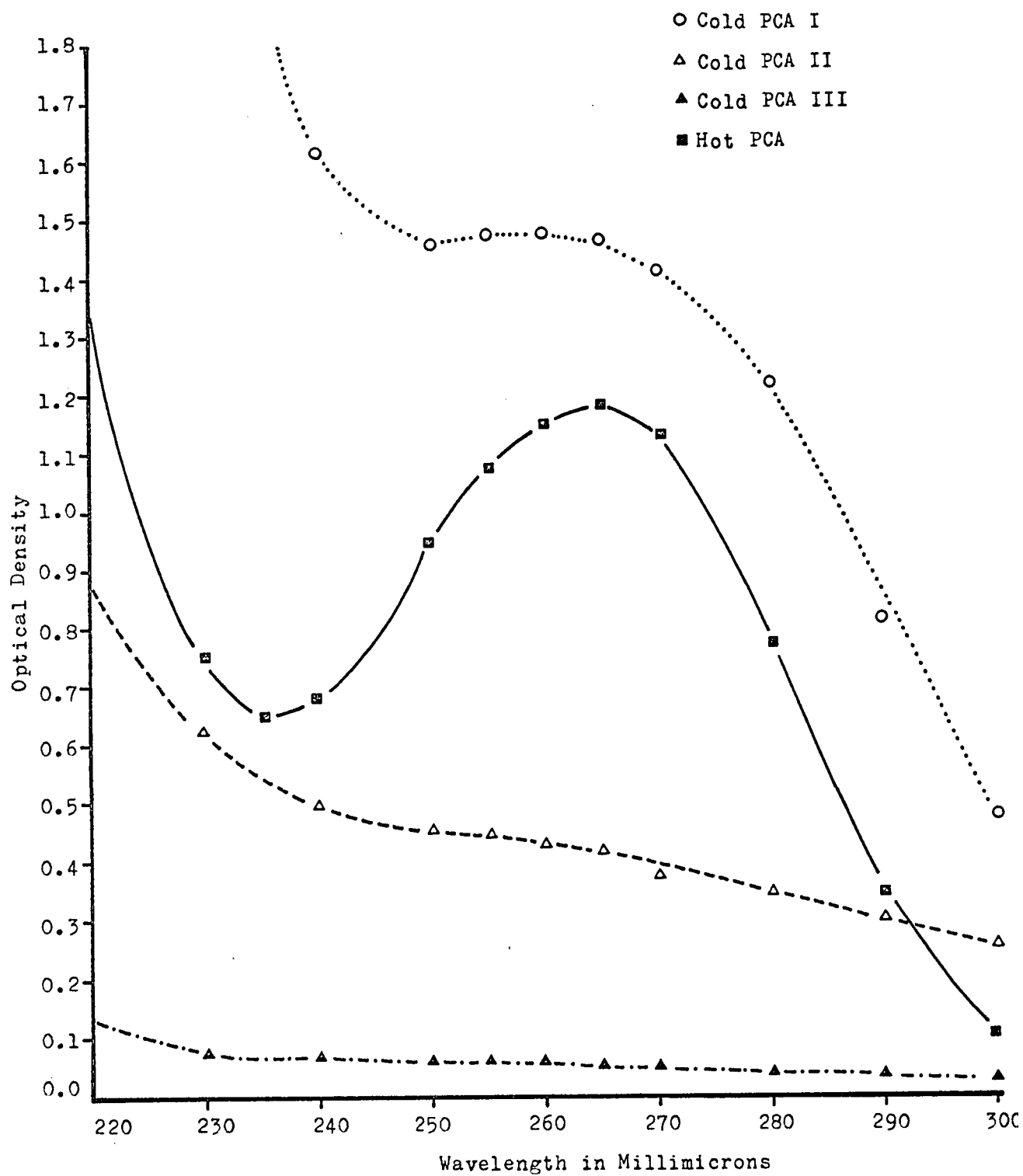


FIGURE 19: Perchloric acid extraction of nucleic acid.

TABLE XVDetermination of Nucleic Acid in a Purified Phage PreparationOrcinol Reaction

Sample	O.D. <sub>665mμ</sub>	μg RNA Equivalents		
		μg	Correction Factor For Dilution	μg/Total Phage
Cold PCA III	0.129	16	x2/3x5	53
Hot PCA	0.605	75	x2/3x3	150
50 μg RNA	0.35			

Diphenylamine Reaction

Sample	O.D. <sub>600mμ</sub>	μg DNA Equivalents		
		μg	Correction Factor For Dilution	μg/Total Phage
Cold PCA III	0.0	0	x5	0
Hot PCA	0.145	35	x3	105
50 μg DNA	0.19			

washing. The hot PCA extract, however, definitely succeeded in removing a fraction insoluble in cold PCA and this fraction had a u.v. absorption spectrum typical of that of nucleic acids or their bases. With the knowledge that one  $\mu\text{g}$  of nucleic acid provides an O.D. reading at 260  $\text{m}\mu$  of 0.03, the amount of nucleic acid present in the hot PCA extract was estimated as being 39.6  $\mu\text{g}/\text{ml}$ , or, for the entire sample of three ml, 118.8  $\mu\text{g}$ .

The orcinol test was positive for this material with  $\mu\text{g}$  RNA equivalents of 75 in the test material or 50/ $\text{ml}$  of the hot PCA extract. According to this value, there would be a total of 150  $\mu\text{g}$  RNA equivalents for the whole sample. However, the colour produced in this reaction was not the normal shade of green and could better be described as olive. This sample was diluted to produce an O.D. reading equal to that of the control RNA at 665  $\text{m}\mu$  and the spectra of the two orcinol tests were plotted using wavelengths ranging between 500 and 700  $\text{m}\mu$  (Fig. 20). This graph portrayed a marked difference between the RNA control and the hot PCA sample. Although both curves had a peak at 665  $\text{m}\mu$ , the sample curve appeared to peak around 530  $\text{m}\mu$  and possibly 600  $\text{m}\mu$  as well. There was obviously some substances other than ribose interacting with the orcinol reagent. The fact that the cold PCA III material contained a total of 53  $\mu\text{g}$  RNA equivalents and yet did not absorb u.v. light at 260  $\text{m}\mu$  suggested that the orcinol reacting material

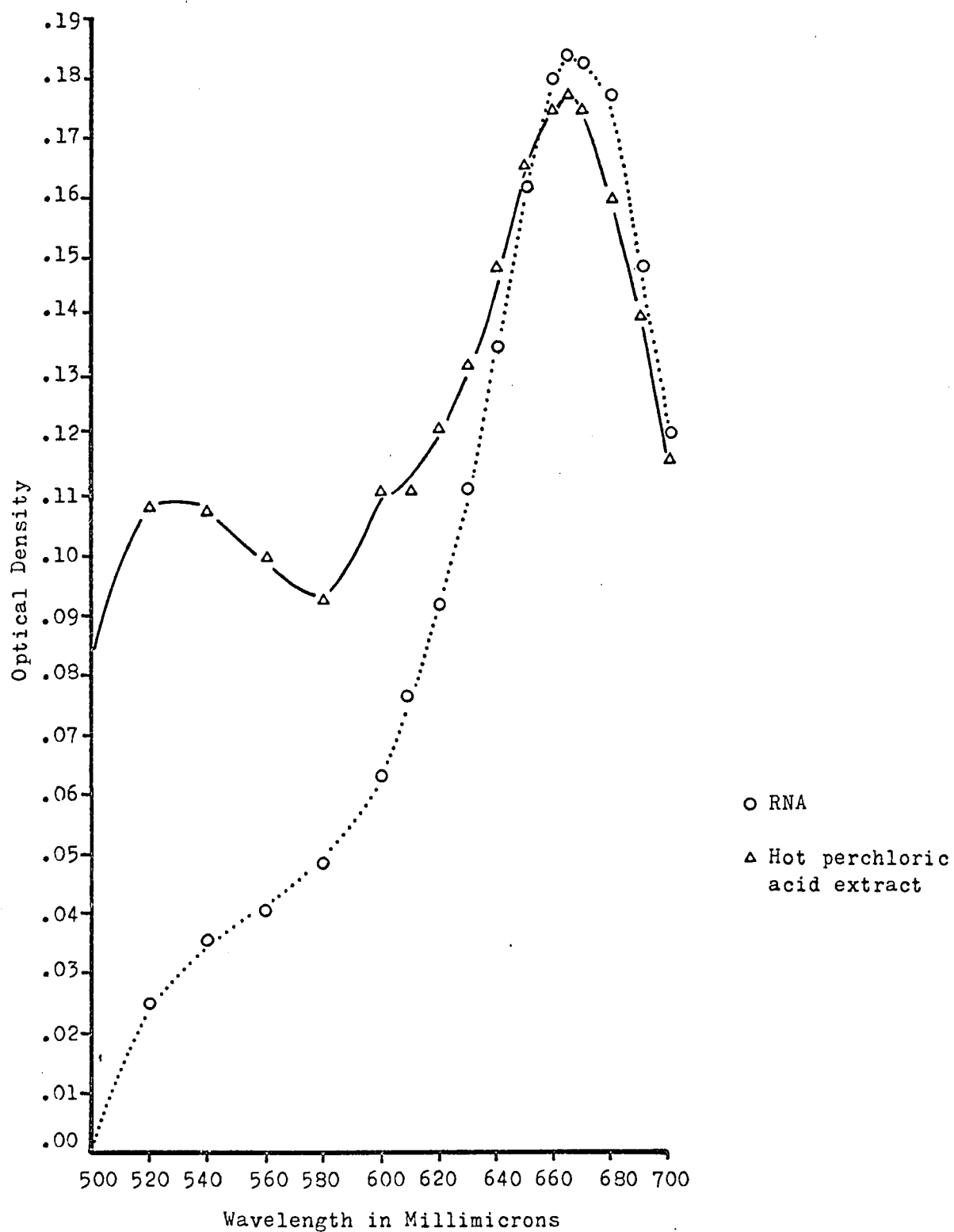


FIGURE 20. Spectrophotometric comparison of orcinol reactions utilizing RNA and the hot perchloric acid extract.

was not RNA or nucleotides. The orcinol reacting material present in the hot PCA extract may also not be RNA.

The diphenylamine reaction demonstrated the presence of 35  $\mu\text{g}$  DNA equivalents in the hot PCA extract, and a total of 105  $\mu\text{g}$  DNA equivalents for the whole sample. These figures were very close to those obtained by estimating the total nucleic acid from the O.D. at 265  $\text{m}\mu$ . Ogur and Rosen (1950) have pointed out that treatment of DNA with hot PCA shifts the peak of maximum absorption from 260  $\text{m}\mu$  to 268  $\text{m}\mu$ . The fact that the hot PCA extract had its maximum u.v. absorption at 265  $\text{m}\mu$ , rather than at 260  $\text{m}\mu$ , suggested that the nucleic acid was DNA.

The acid insoluble material (protein?) in the preparation was of some interest in relation to the above experiments. One ml of 1 N-NaOH was added to the precipitate to dissolve the material, assuming that it was protein; however, the substance did not dissolve over a number of hours of exposure. A few fragments of this material were treated with 10 N-NaOH without effect; therefore, a portion of the 1 N-NaOH preparation was taken and treated with the orcinol reagents. The orcinol test was strongly positive, with a 38.5  $\mu\text{g}$  RNA equivalent reading. A diphenylamine test performed on another portion of the same material was negative. It is possible that the orcinol-reacting material obtained in the hot PCA extract was due either to incomplete separation of

this material from the supernatant fluid or, perhaps, partial hydrolysis of this substance in the hot PCA.

#### Culture Control Experiment

Although a medium control was incorporated into earlier experiments, it was thought that a control experiment should be performed at this time which involved the treatment of a broth culture with flash evaporation and differential centrifugation.

Six 600 ml volumes of broth were inoculated with 2.5 ml each of an overnight culture of strain 13 and incubated for 3-3½ hours. After sedimentation of the bacteria by centrifugation, the supernatant fluid was removed and the sedimented cells were collected and placed in a mortar. The cell paste was then frozen and ground with a pestle after adding an arbitrary quantity of aluminum oxide. Grinding continued until a thick moist paste was obtained. This material was resuspended in 100 ml of broth and then centrifuged to sediment the bacteria and debris, after which the supernatant fluid obtained was added to the original 3600 ml supernatant fluid. The entire volume was filtered through a Millipore GS 0.22 µm membrane filter. This fluid was subsequently treated as previously recorded in Fig. 11. The final precipitate, which resembled that of the phage preparation, was resuspended in two ml of 0.85 per cent saline.

To one ml of the above preparation was added 0.1 ml of ribonuclease and deoxyribonuclease (50  $\mu$ g/ml final concentration for each enzyme). To the other ml of material was added 0.2 ml of 0.85 per cent saline. Both were incubated at 37° C for 30 minutes and then were extracted for their nucleic acid content. Since smaller volumes of test material were involved, the hot PCA extraction was done with 1.5 ml of acid rather than the usual 3 ml and the orcinol and diphenylamine reactions were performed using one-half the normal test volumes and reagent volumes.

The hot PCA extract of the sample not treated with additional enzyme suggested the presence of 15  $\mu$ g RNA equivalents for the total sample, while the enzyme treated sample had a total of 8  $\mu$ g RNA equivalents. It is doubtful if either reading represented RNA since ribonuclease should completely destroy bacterial RNA. However, if the 15  $\mu$ g amount was of significance, it might explain the RNA reading obtained with the phage experiments since nine times this volume of preparation was used in these experiments. Nine times the above reading could provide a total reading of 135  $\mu$ g RNA equivalents; that obtained in the phage experiments was 150  $\mu$ g RNA equivalents.

In this control experiment an attempt was made to disrupt bacterial cells in view of representing lysis of bacteria as might be caused by the phage. It is not known how successful



such disruption was. Since there is no marked lysis of the bacterial culture when the phage is propagated, it would be very difficult to approximate the degree of cell destruction and subsequent release of bacterial nucleic acids.

#### Nucleic Acid Determination by Acridine Orange

Acridine orange has been utilized as a differential stain for DNA and RNA. This dye, when complexed with the appropriate nucleic acid, will fluoresce a characteristic colour when exposed to u.v. irradiation at  $2537 \text{ \AA}^{\circ}$  wavelength. Bradley (1966) described a relatively simple technique for the identification of nucleic acids in viruses. This method has been employed in the study of the nucleic acid of the temperate Cl. perfringens bacteriophage (see Appendix II).

In the following experiments  $1000 \mu\text{g/ml}$  solutions of DNA and RNA were used as controls. It was found that when samples of DNA and RNA were spotted on slides, dried and fixed in Carnoy's fluid, the preparations were washed off the slides. Nucleic acid samples also failed to adhere to the slide with gentle heat fixation; therefore, a small amount of bovine serum albumin (about 0.5 per cent, w/v) was added to the nucleic acid samples before applying them to the slide. These preparations were then gently heat fixed and stained with acridine orange. In these control

slides DNA showed a bright greenish-yellow colour and RNA a flame-red colour when exposed to u.v. light.

When phage preparations were fixed in Carnoy's fluid, they were always lost in the last step of the staining procedure involving  $\text{Na}_2\text{HPO}_4$  treatment; fixation with methanol overcame this problem. A 7-8 drop sample of a concentrated and purified phage preparation containing  $1 \times 10^{10}$  PFU/ml was applied to a slide with a 20  $\mu\text{l}$  capillary pipette, dried, fixed in cold methanol and stained with acridine orange. While still wet with  $\text{Na}_2\text{HPO}_4$  the slide was examined under the ultraviolet lamp. A bright greenish-yellow fluorescence was readily observed similar to that of the control DNA. Such fluorescence might also indicate the presence of double stranded RNA, although no bacterial virus has ever been shown to possess such a nucleic acid.

#### Mitomycin C Experiments

Mitomycin C is an antibiotic which has the property of inhibiting DNA synthesis, while not the synthesis of RNA. Cooper and Zinder (1962), using mitomycin C, have shown selective inhibition of the propagation of a bacteriophage containing DNA, whereas the propagation of a RNA phage was not inhibited. It was thought that the activity of this antibiotic on the propagation of the Cl. perfringens phage might further elucidate the nature of the nucleic acid.

Minimum Inhibitory Concentration (MIC) of Mitomycin C

Mitomycin C was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The label on the vial stated that the contents contained 2 mg of mitomycin C; therefore, the entire contents were resuspended in 40 ml of broth to obtain a stock solution of antibiotic containing 50  $\mu\text{g}/\text{ml}$ .

A tube dilution test (Power, 1955) was performed to determine the MIC of the antibiotic when Cl. perfringens was used as the test organism. One ml of broth was added to each of eight small test tubes. To the first tube was added 1 ml of a 10  $\mu\text{g}/\text{ml}$  solution of mitomycin C and serial halving dilutions were made through tube 7. Tube 8 served as a control. An overnight culture of strain 13 was diluted 1:1000 in broth and 0.1 ml of this dilution was added to each tube. The tubes were incubated under anaerobic conditions in a Brewer's jar.

The MIC was 0.313  $\mu\text{g}/\text{ml}$ . It was noted that the growth of the bacteria was either completely inhibited by the antibiotic or not inhibited at all.

The activity of this antibiotic was very dependent upon the inoculum size. The number of viable bacteria/ml in the antibiotic containing tubes in the above experiment was approximately  $1 \times 10^3$ . When an inoculum of  $1-2 \times 10^7$  viable bacteria/ml was employed, a concentration of 20-40  $\mu\text{g}/\text{ml}$  of mitomycin C was

required to inhibit growth as determined by O.D. readings in a Spectronic 20 spectrophotometer (Fig. 21). In these experiments 0.6 ml of an overnight culture of strain 13 was added to 15 ml of broth containing different concentrations of antibiotic. The cultures were incubated in a water bath at  $37^{\circ}$  C and O.D. readings were made over a five-hour period.

#### Inhibition of Phage Multiplication

Four ml of broth was added to each of six sterile spectrophotometer tubes. To the first tube was added 4 ml of a  $40\text{ }\mu\text{g/ml}$  solution of mitomycin C, and serial halving dilutions were made through tube 5. Due to a slight dilution error in tube 5, the final concentration of antibiotic in this tube was  $1.1\text{ }\mu\text{g/ml}$  rather than  $1.25\text{ }\mu\text{g/ml}$ . Tube 6 served as a control. The tubes were then briefly boiled and cooled and 0.1 ml of an overnight culture of strain 13 was added to each. A very gentle mixing of the cultures was performed by slowly rimming the inside of the tubes with a Pasteur pipette. Readings were made with a Spectronic 20 spectrophotometer at a wavelength transmission of 660 m $\mu$ . Incubation of the tubes was carried out in a water bath at  $37^{\circ}$  C and, after one hour of incubation, 0.5 ml of phage in broth containing  $1 \times 10^5$  PFU/ml was added to each tube so that the final phage titer was approximately  $1 \times 10^4$  PFU/ml. Incubation

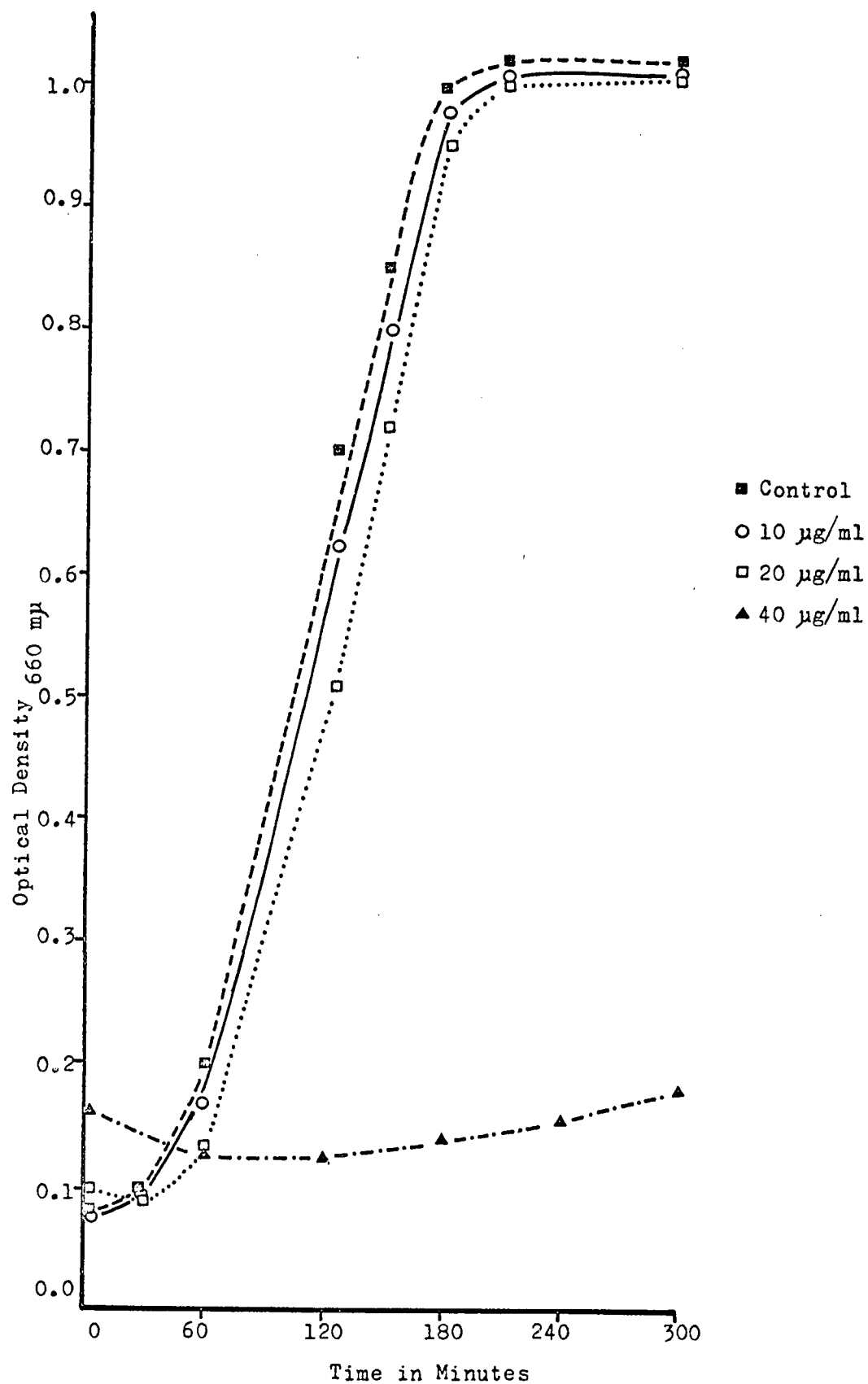


FIGURE 21: Effect of Mitomycin C concentration on the growth of Cl. perfringens.

continued for  $2\frac{1}{2}$  hours and O.D. readings were made at various intervals. After incubation, the bacteria were sedimented by centrifugation and the supernatant fluids were assayed for phage.

The results are recorded in Fig. 22 and Table XVI. Because the O.D. readings obtained for the cultures treated with 10 and 20  $\mu\text{g}/\text{ml}$  of mitomycin C were the same as those obtained for the cultures treated with 2.5 and 5  $\mu\text{g}/\text{ml}$  (no observable bacterial growth), phage assays were not performed.

TABLE XVI

Effect of Mitomycin C on Bacteriophage Multiplication

Sample	Titer PFU/ml	Per Cent Yield Based on Control
Control	$1.4 \times 10^5$	100
1.1 $\mu\text{g}/\text{ml}$	$7.2 \times 10^6$	5,142.8
2.5 $\mu\text{g}/\text{ml}$	$4.5 \times 10^3$	3.2
5.0 $\mu\text{g}/\text{ml}$	$6.0 \times 10^2$	0.4

Both 2.5 and 5  $\mu\text{g}/\text{ml}$  completely inhibited the growth of Cl. perfringens with the inoculum size used in this experiment. A concentration of 1.1  $\mu\text{g}/\text{ml}$  allowed a very limited growth of the organism, but of extreme interest was the observation of a

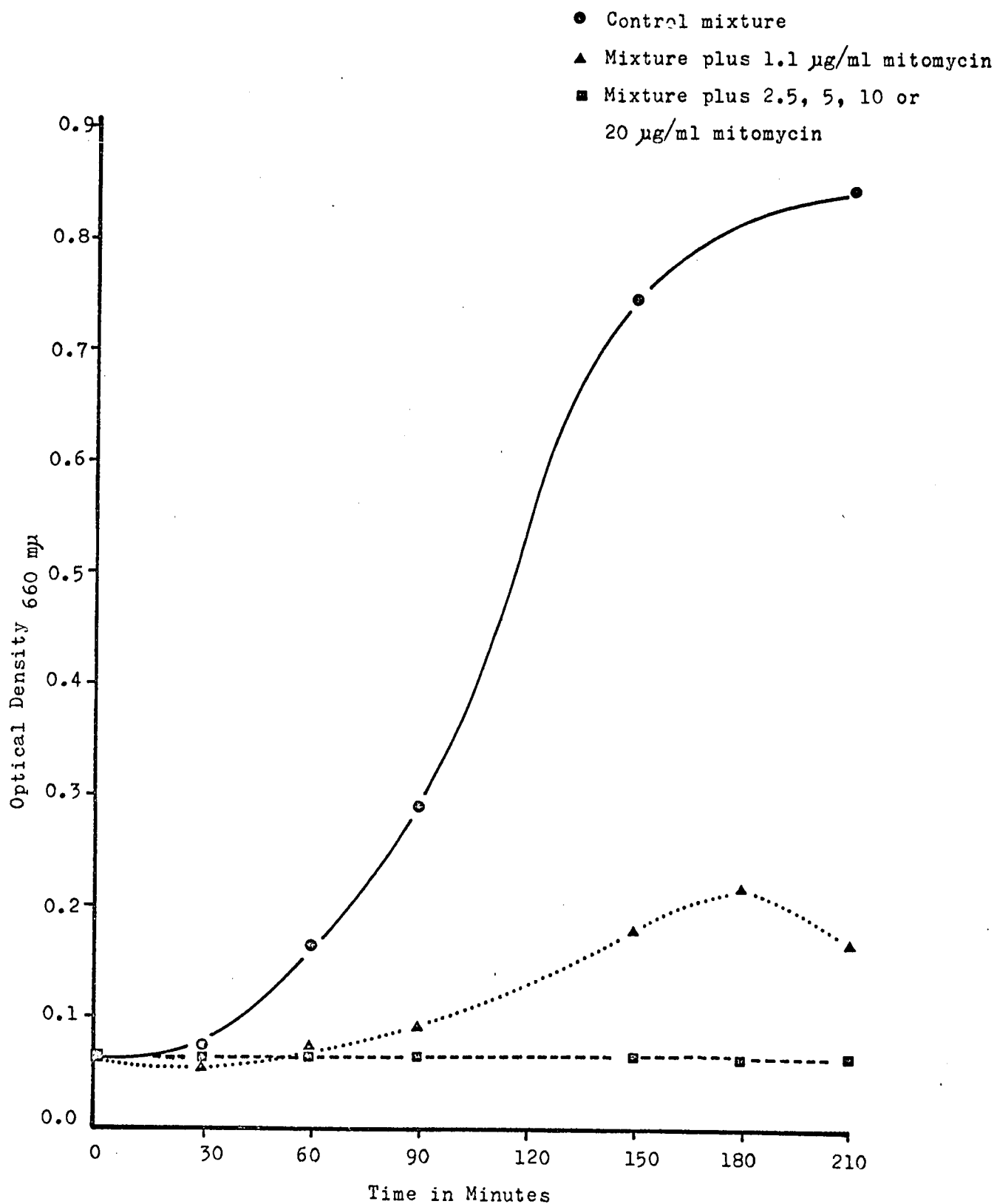


FIGURE 22: Bacterial growth associated with the inhibition of phage multiplication by Mitomycin C.

marked decrease in the O.D. of this culture after 180 minutes of incubation, suggesting lysis of the culture. Associated with this phenomenon was the fact that the phage titer of this culture greatly exceeded that of the control culture (about 50 times). It would seem that this phage infection, in the presence of a low concentration of mitomycin C, had brought about a lytic response rather than the lysogenic response. Mitomycin C has been shown by others (Otsuji et al., 1959) to be an inducing agent of lysogenic bacteria, and it has also been shown to prevent the lysogenic response when a host strain is infected with a temperate bacteriophage (Levine, 1961).

The bacteriophage assays show that mitomycin C has inhibited bacteriophage replication. A concentration of 2.5  $\mu\text{g/ml}$  reduced the phage yield by 97 per cent. Such a sensitivity of phage replication to mitomycin C strongly suggested that the bacteriophage replication was very dependent on DNA synthesis and that the nucleic acid of the phage was probably DNA.



DISCUSSION

This thesis describes the isolation and characterization of a temperate bacteriophage of Clostridium perfringens. Smith, in 1959, pointed out that lysogenic strains of Cl. perfringens did exist and that such strains could be induced by chemical and physical agents to produce an increased number of phage particles; however, he did not describe or characterize the phages involved. This is the only paper published on lysogeny in Cl. perfringens and, although a few papers have appeared describing virulent phages of this species, a temperate phage has never been described. It was, therefore, of interest to isolate lysogenic strains of Cl. perfringens and to see if there might be some relationship between lysogeny and toxin formation.

The early part of this study involved searching for bacteriophages active on Cl. perfringens in the waters surrounding the Montreal area. Virulent phages for this organism have been found in sewage and polluted river waters by both Russian and French workers; however, attempts to isolate a phage from harbour and river waters in this area were unsuccessful, although such waters were highly polluted. Enrichment techniques involving the addition of water samples to cultures of 17 or more strains of Cl. perfringens were of no avail, nor was the precipitation of proteinaceous material by ammonium sulphate followed by

enrichment. Bacteriophages may have been present in the water samples but, without proper indicator strains, could not be detected. The taking of larger water samples or sampling of raw sewage might have met with greater success.

Upon failing to detect any phage in the water samples, a search for lysogeny amongst the Cl. perfringens strains available was undertaken. A lysogenic strain was found within the group of organisms studied, after exposure of such strains to ultraviolet light. The supernatant fluid of this strain possessed a phage active only on one other strain within this group. In retrospect, this phage could have been found much earlier in the experimental work and without irradiation if the supernatant fluids of young cultures had been tested rather than those of overnight cultures. At first it was not realized that Cl. perfringens grew so rapidly and that the maximum release of phage from the lysogenic strain occurred three hours after incubation. At this time approximately 1000 PFU/ml might be detected whereas with older cultures these phages had adsorbed to dead bacteria and debris to such an extent as to not be detectable in small samples. For the same reason, it would be advantageous to always employ the semi-solid agar overlay method for phage detection where a larger sample is to be utilized.

The plaques of the isolated phage were turbid with a portion of resistant growth in the center of each plaque. The

staining technique which was developed for the photography of the plaques greatly elucidated their morphology. These plaques were characteristic of those produced by temperate phages of other genera on their indicator strains. The center portion of resistant growth usually represents lysogenized bacterial cells. The virulent bacteriophages obtained from McClung produced clear plaques on their indicator strain of Cl. perfringens.

Three papers have appeared in the literature dealing with electron microscopy and bacteriophages of Cl. perfringens. The first of these studies was performed on two bacteriophages by Elford, Guélin, Hotchin and Challice in 1953. One of these phages, designated as M, had a head diameter of 35 mμ and tail dimensions of 120 x 15 mμ. This phage was observed to cause rapid clearing of its indicator strain and to produce very high titers according to these workers. The second phage, W, had a head diameter of 60 mμ and a tail similar to that of the M phage. This phage reportedly did not always clear the bacterial culture, although the titers were relatively high. Bychkov (1964) described another bacteriophage of Cl. perfringens which possessed a structureless spherical head, 50-60 mμ in diameter, and a short tail, 15-20 mμ in length. The most recent paper involving a bacteriophage of this species has dealt with a study of a bacteriophage isolated by Guélin in 1947 (Vieu, Guélin and Dauguet, 1965).

This phage was 70  $\mu$  long with a polyhedric head 40  $\mu$  in diameter and a short tail 30  $\mu$  long. The tail portion was unique in that it possessed what appeared to be a contracted end-plate 8  $\mu$  long and 34  $\mu$  in diameter containing 4-7 distinct structures. The tail core which projected beyond this plate was consistently demonstrated in all preparations.

The temperate phage isolated in this study from the lysogenic strain differs considerably from any of the above descriptions. Its tail (200  $\mu$ ) is longer than that of the other isolates and the head structure (80-70  $\mu$ ) is somewhat larger. The head appeared to have a rigid hexagonal profile as shown in the photomicrographs. It may be icosahedral in structure, although studies were not conducted to confirm this supposition. No tail fibers or tail sheaths could be observed, although Dr. Murray pointed out that the technique employed in preparing the material for the electron microscope might not preserve tail fibers, if they should be present. However, the description of this phage resembles that of many temperate bacteriophages of aerobic bacteria, which also appear to have a very simple tail structure. This phage, then, differs from that of previously studied bacteriophages of Cl. perfringens.

Of extreme interest is the fact that the temperate bacteriophage of Cl. perfringens is almost identical, morphol-

ogically, to a temperate bacteriophage isolated from a lysogenic strain of Cl. histolyticum by Guélin, Beerens and Petitprez in 1966. Both of these phages have rigid hexagonal head structures of similar dimension and both possess long straight tails which portray a great number of striations. There is an end-plate about 30 mμ in diameter associated with the Cl. histolyticum phage, although its structure in the photomicrographs was very vague. One picture of the Cl. perfringens phages suggested a similar structure, but since a number of other pictures of the phage did not possess this feature, it was considered an artifact.

Propagation of the temperate bacteriophage in broth or on solid media was successful, although the titers obtained were generally low. Broth culture propagation was the most successful technique with a maximum titer of  $5 \times 10^8$  PFU/ml being obtained. No clearing of the broth ever occurred. Guélin found that one of her phage isolates from river water also produced low titers and did not lyse the bacterial culture in broth. Perhaps such a phage was temperate in nature. The virulent phages obtained from McClung completely lysed their indicator strain in broth within 2-3 hours with final titers in the order of  $4 \times 10^9$  PFU/ml. Since these phages were studied in the laboratory under the same conditions as the temperate phage, it appeared that the difference in the type of response between the two phage types

was quite real and not due to unsatisfactory growth conditions for the Clostridia or phage.

Propagation of the temperate phage on solid media was not a satisfactory means of obtaining high titer lysates. The yield obtained from a plate demonstrating confluent lysis did not exceed that obtainable in broth, and the small volume obtained from such preparations rendered such a method inefficient. The expressed fluid from frozen and thawed plates produced the best yields in this type of experiment. Perhaps the use of plates of greater diameter, rather than the standard Petri dish, would have allowed a higher yield by this method.

The indicator strain 13 grew very rapidly in broth with a maximum viable count being obtained in three hours after inoculation under the conditions of these experiments. Brain heart infusion broth containing 0.1 per cent of sodium thioglycollate was found to be an excellent medium for the growth of Cl. perfringens. Although higher temperatures of incubation were tried for this organism (as suggested by McClung), it was found that 37° C was satisfactory for growth, and this temperature was utilized throughout the experimental procedures. Some preliminary experiments did employ higher temperatures of incubation (41-42° C) and often older cultures were used for phage propagation, but such techniques were abandoned as soon as the nature

of the indicator's growth pattern was determined. It is interesting to note that there was a very rapid fall in the viability of this strain immediately after the peak of maximum viability had been obtained. Since the pH had only dropped to 6.5 at this point, it would not seem that pH was a critical factor in such a response. It would seem difficult to propose that the nutrient content of the medium had been depleted in this short time. Perhaps the bacterial cells were much more sensitive to oxygen at this point and were killed upon plating. No further studies were pursued concerning this phenomenon. It was obvious, however, that a very young culture of the indicator strain had to be utilized for phage propagation.

The state of lysogeny in strain 9 was demonstrated by the fact that after several subcultures of isolated colonies on blood agar plates the colonies chosen for study were all capable of releasing bacteriophage when grown in broth. It was also shown that the lysogenic strain was immune to its own phage since no plaques were formed when this strain was used as an indicator. It was possible to induce the lysogenic strain by the exposure of washed cells to u.v. irradiation. This evidence would suggest that the strain was certainly lysogenic.

An irradiation period of 20-30 seconds was found to produce the greatest induction, whereas a longer exposure period

was far less effective. It would seem that some very critical level of irradiation must exist between 30 and 40 seconds of exposure which either destroys or damages the prophage nucleic acid or seriously disrupts that of the host bacterium. It was found that without diluting the irradiated cells, no induction could be observed. It was thought that perhaps better anaerobic conditions existed if a relatively small inoculum was diluted in a large volume of broth. The phenomenon of readsorption of released phage particles might also be decreased by dilution, although it was unlikely that readsorption could completely mask the induction burst in undiluted cultures. The presence of oxygen was shown by Gáspár (1960) to prevent multiplication of a virulent bacteriophage of Cl. perfringens; therefore, more suitable anaerobic conditions might play a role in this dilution phenomenon.

Induction of the lysogenic strain was also used as a means to produce high titer lysates, but without much success. The actual burst of the induced culture was much greater than that of a lytic cycle of infection, but the dilution necessary for the induction to be realized defeated the higher titers which might be obtainable. Other inducing agents were not used in these experiments. Smith (1959) showed that nitrogen mustard was a very efficient inducing agent for lysogenic strains of Cl. perfringens. Mercaptoacetate (thioglycollate) had poor



inducing qualities and urethane, colchicine and hydrogen peroxide had no effect on the liberation of phage particles in the lysogenic strains, according to Smith. Since Smith required 4 mg/ml of thioglycollate to cause even a ten-fold increment in phage, it was thought that the 1 mg/ml amount present in the broth used in the present experimental work was not acting as an inducing agent, although the effect of this chemical on the lysogenic strain and phage production, in general, was not studied.

It appeared that the indicator strain 13 was lysogenized by the phage at a high multiplicity of infection (m.o.i.). When a concentration of phage greater than that necessary to produce confluent lysis of the indicator strain on solid medium was employed, a confluent lawn of bacterial growth occurred. This growth could not represent phage resistant bacteria, since one would expect to find at least a few resistant colonies present on plates demonstrating confluent lysis. Kinoshita and Teramoto (1955) reported this same phenomenon when studying a temperate bacteriophage of Cl. acetobutylicum. It was found that the resistant growth of Cl. perfringens, when picked and subcultured, could no longer act as an indicator strain, suggesting that it was now immune. Attempts to induce this now resistant strain failed. If indeed the indicator strain was now lysogenic, it was not inducible. The strain appeared to respond as the lysogenic

strain 9 did when irradiated, with its marked lag in growth in broth as compared with a control, but no phage particles could be recovered. It would be interesting to know if this strain was releasing some type of defective phage which could no longer infect the sensitive indicator strain.

An interesting difference existed between the lysogenic strain and the indicator strain. This was the absence of a haemolysin for sheep red blood cells in the lysogenic strain. It was noted that the lysogenic strain was also not pathogenic for the guinea pig, whereas the indicator strain produced typical gas gangrene in this animal. It would be interesting to know if the missing haemolysin was the factor responsible for the lack of virulence of this strain and, also, if the bacteriophage (or prophage) was in any way responsible for the above observations. If phage were responsible for toxin repression (as shown to be possible in staphylococci by Winkler, de Waart and Grooten, 1965) in the lysogenic strain and a conversion phenomenon were to exist, then the phage should be able to prevent toxin formation in the indicator strain. An experiment was designed in which the indicator strain was lysogenized by a high m.o.i. and then the indicator strain was plated over a number of subcultures on sheep blood agar. No non-haemolytic colonies were ever observed. If transduction were involved the frequency of genetic transfer would be much

lower and probably would not be detected by such a crude experiment. A thorough study of what role the phage might play in toxin formation should be pursued.

In the literature it has been suggested that a higher multiplicity of infection encourages the lysogenic response rather than the lytic response. With the Cl. perfringens phage, a very low m.o.i. was essential to observe a maximum lytic response. With a high m.o.i. virtually no phage multiplication could be observed. Since the same number of bacteria was used in each of these tests, it could not be assumed that readsorption was occurring to explain the low yield with high m.o.i. It may be that lysogeny is occurring with the higher m.o.i.

The one-step growth curve of this phage followed the usual pattern of such experiments with a characteristic latent period and release period. After a plateau was reached a second burst was observed to follow. The latent period, 45 minutes, was the same as that observed by Gáspár and Tolnai (1959) for one of Guélin's virulent phages. Guélin (1949) had found a latent period of 15-20 minutes with one of the virulent phages, but it is impossible to determine from the coding system employed by Guélin whether both workers were studying the same phage. The phage yield for the temperate phage was quite low and was also variable. It was not known what the percentage of lysogenization

might be with this phage; therefore, the yield obtained probably is of little significance in terms of number of phage particles released per bacterium. The burst obtained from an induced lysogenic culture was very large. A prolonged latent period was observed in this experiment, a characteristic of induced phage.

The purification and concentration of the clostridial phage was rather difficult. The phage seemed to be very sensitive to high speed centrifugation. Lower speeds were not successful in concentrating the phage while higher speeds resulted in up to 90 per cent loss in phage titer. Eventually one speed was found which succeeded in concentrating the phage seven times, although 70 per cent of the phage titer was lost. Perhaps some yet slower centrifugation speed or shorter period of centrifugation might improve the phage yields. It was conjectured that the loss in phage titer under these conditions might be due to the breakage of the phage tail. Some of the electron photomicrographs did suggest that such might be the case since a number of phage heads had been found separated from the tails; however, it is not known at what point this damage might have occurred.

Concentration of lysates by flash evaporation was very successful in increasing the phage titer, but undesirable portions of the broth were also concentrated. The magnesium pyrophosphate gel method of phage purification suggested by Schito (1966) worked

very well, although there was only a small concentration of phage particles. It was felt that this method would be an excellent means of preparing a reasonably pure stock of phage for experimental work. It should be noted that the gel could adsorb bacterial nucleic acid as well as some components of the medium. Flash evaporation of the gel eluate was unsatisfactory since these contaminating elements were concentrated to objectionable levels in the process.

High speed centrifugation of the gel eluate was not considered practical in terms of obtaining concentrations in the order of 100-fold. As it was intended to perform nucleic acid determinations on this phage, high titer lysates of reasonable purity were desirable. Since centrifugation yields were of such a low order, it was essential to first increase the titer of the broth lysate about ten-fold before centrifugation was performed. Flash evaporation was the only technique which was successful in this case, followed by two cycles of high speed differential centrifugation.

The temperature sensitivity of this bacteriophage proved to be very interesting. Although temperatures of 60 and 70° C inactivated the phage very quickly, temperatures of 40 and 50° C served to increase the phage titers up to ten times that of the unheated control. This phenomenon only occurred with a semi-

purified phage preparation maintained in phosphate buffer containing magnesium sulphate. It could not be shown what factors were involved in the heat activation but experiments were designed to determine whether clumping of phage particles might be involved, magnesium ions were responsible or a repressor substance might be present. It was interesting to note that a similar situation involving Reovirus was observed by Wallace, Smith and Melnick (1964) and that these workers could not find an explanation for this peculiar activity. Magnesium chloride appeared to be essential in their experiments.

Magnesium ions in the clostridial phage experiments were shown not to be responsible for heat activation, but rather to provide considerable protection against heat inactivation. It was also shown that in the presence of one molar magnesium sulphate, phage titers were increased over those of control samples, suggesting that magnesium might play a role in adsorption or the infective process.

The pH sensitivity of the phage did not demonstrate any peculiarities. A pH of 3 completely inactivated the phage whereas a pH of 8 and 9 appeared to enhance the titer over control values, although this enhancement was not particularly significant.

The u.v. inactivation of the phage portrayed a first order reaction.

Adsorption experiments revealed that the phage was adsorbed to the indicator strain at a rapid rate within the first two to four minutes when approximately 50 per cent of the PFU had been adsorbed, after which adsorption continued at a decreased rate until 70-75 per cent of the PFU were adsorbed by 20 minutes. It is not known why the rate of adsorption decreased after the initial adsorption velocity observed. It might be proposed that there is a limited number of adsorption sites available on the bacterial cells. However, the m.o.i. is so low in these experiments that such an explanation may not be valid. It is likely that when the phage particles are first added to the culture that the majority of them come into ready contact with the bacterial cells. After the initial mixing, there is no further agitation of the mixture and it would seem reasonable to suggest that the rate of phage-bacterium interaction would decrease in such an environment.

The nucleic acid determinations of this Cl. perfringens phage were difficult due to the low titer lysates produced by the phage and the complexity of the broth medium in which the phage was suspended. Flash evaporation of the broth lysate followed by two cycles of differential centrifugation provided a relatively pure preparation of high titer. The hot perchloric acid extract of this material revealed the presence of nucleic acid as shown

by the u.v. absorption curves plotted for this material. This extract, however, possessed both diphenylamine reacting and orcinol reacting substances. Since the phage preparation had been treated with ribonuclease and deoxyribonuclease before centrifugation, it was unlikely that any bacterial nucleic acids would be present in this sample. The total amount of nucleic acid in the sample as estimated from the amount of u.v. light absorption at 265  $m\mu$  was very close to the amount of DNA as determined by the diphenylamine reaction for the same material. The fact that the peak of maximum u.v. absorption falls at 265  $m\mu$  rather than at 260  $m\mu$  is characteristic of DNA treated with hot perchloric acid (Ogur and Rosen, 1950). In this experiment the cold acid extracts contained orcinol-reacting materials although one of these extracts demonstrated no absorption of u.v. light at 260  $m\mu$ . Therefore, the test material contained orcinol-reacting materials not associated with RNA. This fact, of course, can not preclude that the hot perchloric acid extract did not contain some RNA, but it does give evidence that the orcinol test is rather unreliable for the sole detection of RNA-associated pentose. The hot acid-insoluble residue from this extraction procedure also gave a strong orcinol reaction, but no diphenylamine reaction. The diphenylamine reaction is a well established specific and sensitive tool for the measurement of DNA whereas a considerable number of carbohydrates can



react with the orcinol reagent (Ashwell, 1957). Zimmerer, Hamilton and Pootjes (1966) were evidently confronted with a similar problem in characterizing a temperate bacteriophage of Agrobacterium tumefaciens. The diphenylamine test on the nucleic acid was clearly positive, whereas the orcinol test gave inconclusive results.

Acridine orange staining of the phage particles demonstrated that DNA was the nucleic acid in the phage. The only other nucleic acid which would give the same fluorescence, according to Bradley (1966), would be double stranded RNA. Such a nucleic acid has not been found in a bacteriophage and is a very rare occurrence in non-bacterial viruses. It would be difficult to fit a RNA phage into the role of lysogeny and its association with the DNA of the host genome.

It was shown that multiplication of the phage was inhibited with low concentrations of mitomycin C, a fact suggesting that the phage was very dependent on DNA synthesis. Such a test was shown by Cooper and Zinder (1962) to differentiate a RNA bacteriophage from one possessing DNA. The multiplication of the RNA phage was not inhibited by this antibiotic in their experiments, even though the host organism appeared to be killed by the antibiotic.

Therefore, in view of the above evidence, it would seem reasonable to conclude that the nucleic acid in this temperate bacteriophage of Cl. perfringens is DNA.

The characterization of this phage has revealed that there are no major differences in the chemical or physical properties from those properties of phages active on aerobic bacteria. Lysogeny has been shown to exist in this species of Clostridium, as pointed out by Smith, and the lysogenic strain studied in this experimental work has been shown to be inducible in a manner similar to that of lysogenic aerobic bacteria.

SUMMARY

Preliminary attempts to isolate a bacteriophage for Clostridium perfringens from river water were without success; however, in screening 21 strains of this organism for lysogenicity, one lysogenic strain and one indicator strain were discovered. The lysogenic strain was shown to be induced by a 20 second exposure to ultraviolet light and to be resistant to its own phage. The lysogenic and indicator strains differed in that the lysogenic strain lacked a haemolysin for sheep red blood cells and was not pathogenic for guinea pigs, but in the few preliminary experiments performed, no relationship between phage and haemolysin activity could be observed.

Growth of the Clostridia and propagation of the phage were successful in brain heart infusion broth containing 0.1 per cent sodium thioglycollate. The indicator strain was shown to grow very rapidly at 37° C with the peak of its growth curve occurring after three hours of growth. Young cultures were therefore used for the propagation of the phage. These cultures were never lysed when the phage was propagated and the maximum titer obtained was  $5 \times 10^8$  PFU/ml. Lab Lemco agar was found to be an excellent solid medium for phage assays and the plaques formed in the semi-solid agar overlay ranged from pinpoint to 1.5 mm in diameter. A staining technique was developed for the

photography of these plaques, which clearly defined resistant growth in the center of the plaques. Lysogenization of the indicator strain was believed to occur.

Electron microscopy revealed that the phage had a polyhedric head 80 x 70 mμ in diameter and a tail possessing many striations, which was 200 mμ long. No tail sheaths or fibers were observed.

It was shown that a low multiplicity of infection was required to obtain a maximum burst. One-step growth curves revealed latent and release periods of 45 minutes each. These features were very consistent, but the yield obtained varied considerably.

Various means of purifying and concentrating the phage preparations were attempted including centrifugation, adsorption of the phage to a magnesium pyrophosphate gel with subsequent elution, flash evaporation and differential centrifugation. Although all of these methods had their advantages and disadvantages, flash evaporation plus differential centrifugation was found to be the only means employed to obtain a high titer preparation of relative purity. Experiments on temperature sensitivity demonstrated a strange phenomenon of heat "activation" when the phage was exposed to 40 and 50° C. Only a certain preparation of phage portrayed this phenomenon. Although a number of experiments

were performed in an attempt to elucidate the nature of this reaction, no conclusion could be drawn. It was shown, however, that magnesium ions protected the phage considerably against heat inactivation, and might also play a role in phage adsorption.

The phage was shown to be stable over a wide range of pH values. Complete inactivation occurred at pH 3 and there might be a slight increase in phage titer over that of the control at pH 8 and 9.

Ultraviolet light inactivation occurred as a first order reaction, with 99 per cent of the phage in the sample being inactivated in 55 seconds.

Adsorption experiments showed that the phage adsorbed to the bacterial cells very quickly for the first 2-4 minutes, after which there was a decrease in the adsorption rate. About 75 per cent of the plaque forming units was adsorbed by 20 minutes.

The nucleic acid determination of this phage was hindered by the complexity of the medium and the generally low titers of phage attainable in the propagation techniques. The only method of obtaining a high enough titer for the nucleic acid determination was that involving flash evaporation and differential centrifugation of the broth lysates. This technique gave a final titer around  $1-3 \times 10^{10}$  plaque forming units/ml. It should be noted that the phage was readily inactivated by high centrifugation speeds.

The purified preparation was extracted by perchloric acid, using cold and hot treatments, and the nucleic acid was determined by the diphenylamine and orcinol reactions. Both tests were positive, but, for a number of reasons, the DNA determination appeared to be the correct one. Many portions of the extraction procedure were observed to react with the orcinol reagents and this test was considered unreliable for RNA determination.

When a high concentration of phage particles was stained with acridine orange, a fluorescence characteristic of DNA was observed.

The inhibition of the phage propagation by low concentrations of mitomycin C strongly suggested that the phage was a DNA containing phage. Thus, as a result of a number of tests, it was concluded that the phage contained DNA.

CONTRIBUTION TO KNOWLEDGE

This work represented the first characterization of a temperate bacteriophage of Clostridium perfringens. It was also the second report of lysogeny in this species.

This temperate phage was larger than any of the virulent phages studied for Cl. perfringens, as determined by electron microscopy, and its morphology differed considerably from that of two virulent Cl. perfringens phages described by Bychkov (1964) and Vieu, Guélin and Dauguet (1965).

It was demonstrated that brain heat infusion broth containing a reducing agent was a satisfactory medium for bacterial growth and the propagation of phage, and it appeared that techniques involving the bubbling of nitrogen through the culture were not necessary for the characterization of phage growth.

A rapid and simple technique was described for the staining of bacteriophage plaques produced in semi-solid agar.

Methods for the concentration and purification of this phage were developed, including the use of a new technique described by Schito (1966).

"Activation" of the bacteriophage by heat was a property unique to this study, although the significance of the phenomenon was uncertain.

The nucleic acid determination was the first to be performed on a bacteriophage of Cl. perfringens and the second to be performed on any clostridial phage. Mitomycin C and acridine orange were shown to be useful tools in the identification of the type of nucleic acid.



APPENDIX IMedia and BuffersLab Lemco Agar

Proteose peptone (Difco) . . . . .	10.0 g
Yeast extract (Difco) . . . . .	4.0 g
Lab-Lemco Beef (Oxoid) . . . . .	8.0 g
Stock salts solution . . . . .	20.0 ml
Distilled water made up to . . . . .	1000.0 ml

Melt the ingredients (except agar) in 50 per cent of the final volume. Melt the agar in the remaining volume in the autoclave and mix with the other ingredients. Adjust the pH to 7.2 and filter through pulp paper. Bottle and autoclave at 121° C for 20 min. For pour plates, cool to 50° C and pour aseptically 20 ml/plate. Incubate for 24 hour at 37° C as a sterility check. Store the plates at 4° C.

Stock Salts Solution

NaCl . . . . .	250.0 g
KCl . . . . .	20.0 g
CaCl <sub>2</sub> . . . . .	10.0 g
Distilled water made up to	1000.0 ml

Brewer's Meat Medium

Use 16 x 125 mm screw-capped tubes. Add Robertson's meat mash about 2.0 cm up the tube and cover with 10 ml of thioglycollate broth. Steam 30 min at 100° C and autoclave at 121° C for 20 min. Store at room temperature.

Thioglycollate Broth (for Brewer's Medium)

Heart infusion broth (pH 7.6) . . . . . 1000.0 ml

Dissolve in it Bacto agar . . . . . 0.5 g

Add:

Glucose . . . . . 10.0 g

Sodium thioglycollate . . . . . 1.0 g

Check pH for 7.4-7.6. Add 1 ml of a 0.2% aqueous solution of methylene blue.

Blood Agar Slopes and Plates

To 500 ml of molten Lemco agar at 50° C and 25 ml of sterile defibrinated sheep blood. Mix well.

For slopes: Distribute aseptically in 16 x 150 mm sterile tubes, 5 ml/tube.

For plates: Pour aseptically 20 ml/plate. Incubate for 24 hr at 37° C as a sterility check.

Egg Yolk Medium

Dip an egg in alcohol. Flame. Break and collect the yolk in a sterile measuring cylinder. Add an equal volume of sterile 0.85% sodium chloride solution. Emulsify with a sterile pipette.

To 300 ml of molten heart infusion agar at 50° C add 30 ml of the emulsion. Pour plates, 20 ml/plate. Incubate for 24 hr at 37° C as a sterility check.

Phosphate Buffer (0.067 M, pH 7.1) Containing  $2 \times 10^{-3}$  M-MgSO<sub>4</sub>

- A. KH<sub>2</sub>PO<sub>4</sub> . . . . . 0.067 M  
 B. Na<sub>2</sub>HPO<sub>4</sub> . . . . . 0.067 M

Mix 300 ml of A with 700 ml of B to obtain 1000 ml of buffer at pH 7.1-7.2. To 0.4930 g of MgSO<sub>4</sub> added buffer to a volume of 1000.0 ml.

Potassium Phosphate Buffer (0.3 M, pH 7.0)

(For the magnesium gel experiments only)

- A. K<sub>2</sub>HPO<sub>4</sub> . . . . . 0.3 M  
 B. KH<sub>2</sub>PO<sub>4</sub> . . . . . 0.3 M

Mix 334 ml of A with 166 ml of B to obtain 500 ml of buffer at pH 7.0.

Modified McIlvaine's Citric Acid and Phosphate Buffer (pH 3.8)

(For acridine orange staining only)

A. Citric acid . . . . . 0.1 M

B.  $\text{Na}_2\text{HPO}_4$  . . . . . 0.15 M

Add 600 ml of A to 400 ml of B to obtain 1000 ml buffer,

pH 3.8.

## APPENDIX II

### Methods

#### Purification of Bacteriophage (Method of Schito, 1966)

##### Preparation of Magnesium Pyrophosphate (Mg-PPi) Gel

Add 2 vol of 0.1 M-sodium pyrophosphate to 10 vol of a 0.1 M-magnesium chloride solution. Stir vigorously with an electric stirrer. Collect the gel by slow speed centrifugation (1800 r.p.m. in an International UV Centrifuge, Head No. 266) and wash once with distilled water (preferably deionized water).

##### Adsorption

Dialyze a clarified bacteriophage lysate against running tap water overnight. Add the dialyzed lysate to the sediment of a double volume of washed Mg-PPi gel. Stir constantly with a magnetic stirrer at room temperature, but not at too brisk a rate. Allow about 1.5 hours for adsorption (Schito recommends 30 min, but this was not adequate for the C1. perfringens phage). Sediment the gel by slow speed centrifugation and wash three times with distilled water.

##### Elution

After a final packing of the gel by slow speed centrifugation, elute the phage by the addition of an equal volume

of 0.3 M-potassium phosphate buffer (Appendix I). Stir the mixture for 1 hr and then centrifuge to sediment the gel. The supernatant fluid contains the purified and concentrated bacteriophage.

#### Extraction of Nucleic Acid

(This method is based on the Schneider extraction of nucleic acids -Schneider, 1945; Schneider, Hogeboom and Ross, 1950.)

Dilute 70-72% perchloric acid (PCA) in water (v/v) to prepare working concentrations of 10 and 5%. To the material to be extracted, add an equal volume of 10% PCA or an appropriate amount of concentrated PCA to obtain a final concentration of 5%. Chill this preparation at 0-4° C for 30 min and then centrifuge in a refrigerated centrifuge at 12,350 g for 10 min. Wash the sediment twice with cold 5% PCA and keep all washings for spectrophotometric study and/or chemical analysis.

To the washed sediment add 3 ml of 5% PCA and heat at 90° C for 15 min. Centrifuge again and collect the supernatant fluid, which contains the nucleic acid fraction.

The lipid extraction step suggested for tissue cells was omitted in this extraction.

DNA Determination by the Diphenylamine Reaction (Burton, 1956)

Reagents:

- A. 5% PCA
- B. 100 ml glacial acetic acid containing 1.5 g diphenylamine (Fisher) and 1.5 ml  $\text{H}_2\text{SO}_4$
- C. Aqueous acetaldehyde, 16 mg/ml (1 ml acetaldehyde in 50 ml water) Add 0.1 ml to 20 ml reagent B.

Procedure:

To 1 ml of DNA solution add 1 ml of 5% PCA and 4 ml of reagent (B + C). Mix and seal the tubes with parafilm and wrap them in aluminum foil to avoid light exposure. Place in the dark for 16-20 hr at room temperature.

Read in a spectrophotometer at 600 m $\mu$  wave length.

A characteristic blue colour signifies a positive test.

Controls include a 50  $\mu\text{g}$  DNA preparation and a reagent control substituting 5% PCA for the sample.

RNA Determination by the Orcinol Test (Ashwell, 1957)

Reagents:

- A. 100 ml conc HCl containing 0.5 ml of 10%  $\text{FeCl}_3$
- B. 6% orcinol (recrystallized from benzene) in 95% ethanol (e.g. 150 mg in 2.5 ml ethanol for every 10 tubes)

Procedure:

To 1.5 ml of sample add 3.0 ml of reagent A and 0.2 ml of reagent B. Boil for 20 min. Cool in ice. Read in a spectrophotometer at 665 mμ wavelength.

A characteristic green colour signifies a positive test. (If DNA is present, subtract 6% from the values obtained.) Controls include a 50 μg RNA preparation and a reagent control substituting 5% PCA for the sample.

Note: The orcinol reagent (B) must be made up fresh for each experiment.

Fluorescent Staining of Phage Nucleic Acid (Bradley, 1966)Reagents:

- A. Cold methanol (2° C)
- B. Modified McIlvaine's citric acid and phosphate buffer (Appendix I)
- C. Acridine orange 0.01% in 10 ml of B
- D. Disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) 0.15 M

Procedure:

Add small drops of a phage preparation containing  $1 \times 10^{10}$  PFU/ml to a microscope slide by means of a 20 μl capillary pipette,



each drop being dried by a stream of warm air before applying another to the same spot. Fix the slide in cold methanol for 15-20 min, dry and then rinse briefly in two baths of buffer. Place the slide in a bath of  $\text{Na}_2\text{HPO}_4$  for 15-20 min. While the slide is still wet, examine under a u.v. light (UVS-II hand lamp, Ultra-violet Products, Inc. San Gabriel, California) emitting a wavelength of  $2537 \text{ \AA}$  and note the colour of the fluorescence.

Note: Carnoy's fluid (1 part glacial acetic acid, 3 parts chloroform and 6 parts ethanol) is an alternative fixing fluid.

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