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1991
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LYSOGENY IN ENCAPSULATED AND NONTYPABLE STRAINS OF *HAEMOPHILUS INFLUENZAE*

Tshlidzi Nelcus Muthivhi

A dissertation submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

1991

ABSTRACT

Limited information on the prevalence of lysogeny and the morphological features of bacteriophages in encapsulated and nontypable strains of *Haemophilus influenzae* is available from the literature. This study was conducted to investigate lysogeny in wild-type strains of *H. influenzae* and to characterize bacteriophages recovered from these clinical strains. Following induction by mitomycin C, 16 of 58 isolates of *H. influenzae* tested produced lysis. Addition of chloramphenicol inhibited cell lysis, indicating that protein synthesis was essential for induction. Type b capsules did not inhibit cell lysis or their ability to become lysoginized.

Bacteriophage particles were detected by electron microscopy in 11 lysates and three morphological groups, A1, B1 and F were identified. None of these phages was capable of producing plaques when tested on a total of 114 strains from species belonging to the *Haemophilus* or other genera. Five lysates, however, exhibited killing properties involving several nontypable *H. influenzae* strains and this property was retained by sediments after centrifugation, implicating bacteriophage as the killing principle. These phages were regarded as genetically defective by virtue of their inability to produce plaques and the findings of this study suggest that they may exist in a biological form more primitive than temperate phages. The present investigation was the first to demonstrate and characterize a large group of inducible phages in clinical isolates of *H. influenzae*.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the Faculty of Medicine, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Tshilidzi Nelcus Muthivhi

April 1991

Parts of this dissertation have been published in the following paper :

MUTHIVHI, T.N., GROMKOVA, R.C., SHARP, P.E., and KOORNHOF, H.J. 1991. Lysogeny in encapsulated and nontypable strains of *Haemophilus influenzae* .*Current Microbiology* 22

ACKNOWLEDGEMENTS.

It is with sincere appreciation that the following persons are acknowledged :

Professor J. Metz, former Director of the South African Institute of Medical Research (SAIMR), Johannesburg, for the use of laboratory facilities required for this study.

Professor H.J. Koornhof, Head of the Emergent Pathogen Research Unit, for the time he spent reviewing this dissertation with constructive criticism and helpful suggestions.

Dr R.C. Gromkova, my supervisor, for devoting so much of her precious time guiding and advising me in this investigation. This study would not have been completed without her encouragement and understanding. It was a valuable experience to have a supervisor with such a broad understanding and experience in the field of microbial genetics.

Ms P. Sharp for the help given when using the electron microscope and dark room at the Medical School, University of the Witwatersrand.

The SAIMR, Medical Research Council and the University of the Witwatersrand for funding the research project.

Harry and Helen Windsor for their assistance in typing and arranging this dissertation. Their unfailing friendship and moral support is very dear to me.

My parents, brothers and sister for their patience and endless support.

Several special friends for their confidence in my ability to complete this study.

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1. GENERAL INTRODUCTION

1.1. Discovery of bacteriophages

In 1915 Twort described the infectious destruction of micrococcal colonies by an agent that passed through bacteria-proof filters, was inactivated by heating at 60°C for one hour, and could not grow autonomously (Twort,1915). Two years later d'Herelle working at the Pasteur Institute in Paris, published his independent discovery of a similar agent active on Shiga's bacilli and named it a bacteriophage (eater of bacteria). Early hopes of using the action of phages on susceptible bacteria as a means of preventing and treating infectious diseases were not fulfilled.

1.2. Origin of bacteriophages

There have been several hypotheses put forward to describe the origin of bacteriophages (Adams, 1959). The two likeliest hypotheses are the following:

- Bacteriophages (phages) are the direct descendants of primitive form of life that originated prior to the evolution of organised cells.

- Phages are derived from bacterial genetic material that has acquired the capacity to exist and function independently. According to this hypothesis a series of mutations that occurred in a segment of the genetic material of a bacterium has conferred to that segment a certain degree of autonomy. The mutations resulted in the synthesis of a specific kind of protein serving to protect the segment from damage by the extracellular environment and to facilitate the transfer of genetic material from one cell to another. This hypothesis is consistent with the observed fact that the genetic material of certain temperate phages can associate more or less permanently with genetic loci in the hereditary apparatus of the host cell and thus become part of that apparatus. Once temperate phages have envolved they could be irreversibly transformed into virulent phages by further mutations. Host range mutations of a virulent phage might permit it to attack a host with which it had no genetic affinity whatever so ultimately to give rise to a hypervirulent phage that has lost all resemblance to any known strain.

1.3. Nature of the bacteriophage

From the very start of his studies d'Herelle (1917) hypothesised that the phages are probably viruses. This assumption was made on the basis of their ability to pass through bacterial filters and the inability to be seen under the light microscope. Like other viruses phages are infectious and are able to multiply in the cells. D'Herelle's view found few followers among bacteriologists. Bordet and Gratia preferred to regard Twort-d'Herelle's agent as a self-stimulating enzyme endogenous to the bacterium. Most of their experimental support was derived from the study of lysogenic bacteria. Schlesinger, and Delbruck in the 1930s confirmed the viral nature of the Twort-d'Herelle's agent (Stent, 1963).

The development of the electron microscope during the late 1930s finally made possible the direct visualisation of the phage particles and the description of their morphology. A typical phage particle consists of a head and a tail. The head represents a tightly packed core of nucleic acid surrounded by a protein coat or capsid. The protein capsid is made up of identical sub-units, packed to form a prismatic structure, usually hexagonal in cross section. The phage tail varies tremendously in its complexity from one phage to another, with three basic morphological patterns observed commonly. The first, exemplified by coliphages T2, T4 and T6, has a complex tail consisting of at least three parts: a hollow core; a contractile sheath; and a terminal base-plate, to which may be attached tail fibres (Fig 1.1). The second pattern, of which coliphage T1 and lambda (λ), are prime examples consists of a non-concontractile flexible tube with fine fibres protruding from the distal end. The third morphological pattern, as evidenced by coliphage T3 and T7 exhibits very short structure and consists of very few protein species. Some phages lack tails, and the capsid is a simple icosahedron (ϕX174), whereas others are filamentous with capsids displaying helical symmetry (F1).

Although d'Herelle (1926) and Burnet (1929) had concluded, even before 1930, that phages are released from infected cells in a burst that occurs a certain time after infection, there were reports opposing this view which suggested that phage production was a continuous process. Ellis and Delbruck (1939) designed the one-step growth experiment which showed that each phage-infected bacterium liberated several hundred progeny phages after a latent period, and the end of the latent period and onset of bacterial lysis occur at the same time. The one-step growth experiment demonstrated clearly the nature and overall kinetics of the multiplication of bacterial viruses in the bacterial hosts. The experiment, however,

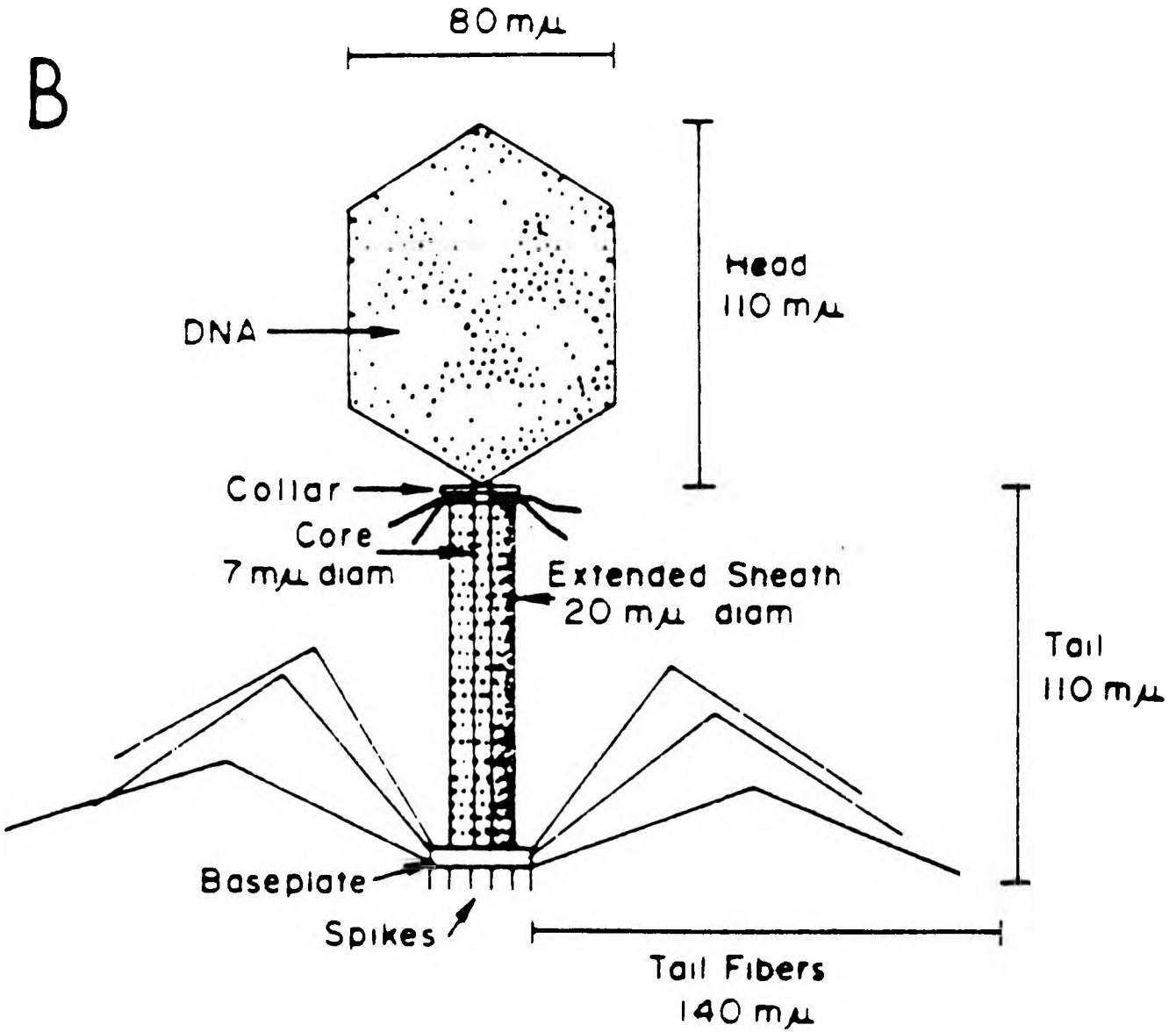


Figure 1.1 Model of phage T4.

said nothing about the existence of intracellular phage before lysis of the cell. Doermann (1952) devised a way to break open the infected cells during the latent period and assayed for the infectivity of the material released at specific time intervals. This experiment showed no infectious particles present during the first half of the latent period. During the latter half of the latent period intracellular phages appeared rapidly. Hershey and Chase (1952) demonstrated that, upon adsorption to the cell surface, the phage dissociates into its two principal parts. The DNA moves to the interior of the cell, while the outer protein coat remained fixed to the cell surface, from which it could be removed without affecting the replication process. Thus the eclipse period discovered by Doermann was explained. The integrity of the phage particle is destroyed shortly after attachment of the phage to the host cell. More importantly, this experiment showed that, after infection, the part of the infecting phage particle which provides genetic continuity to its progeny is its DNA. During the following year Watson and Crick (1953) deduced the double-helical structure of DNA molecule from X-rays diffraction data of T4 phage DNA and pointed out its biological implications. Since then the nucleic acids of many phages have been studied. It has been found that they may exist in several forms. The larger, more complex bacteriophages generally contain a single, duplex DNA molecule. The majority have a linear duplex DNA as is the case of *Escherichia coli* T-phage DNA. Few of these bacteriophages, such as PM2 contain a circular genome. Most of the smaller phage genomes are single-stranded RNA linear molecules as is the case of MS2. There is a phage of *Pseudomonas phaseolica*, $\phi 6$, that contains double stranded RNA (Joklik *et al*., 1988).

In their life cycle bacteriophages pass through a stage in which their genetic substance is the only material link connecting one generation with the next. The parental DNA directs the synthesis of more phage nucleic acid (autocatalytic synthesis) and, in addition, directs the synthesis of the phage proteins (heterocatalytic synthesis). Phage-precursor proteins and phage-precursor DNA are first synthesised separately in the infected cell. During the maturation process, the phage capsid arises by aggregation of a thousand or so identical head protein polypeptide sub-units which thus form their quaternary protein structure. The capsid becomes filled with the phage precursor DNA. This process is called packaging of the heads. Finally, the nascent DNA-filled phage capsid is assembled with a phage tail.

A typical phage may exist in three states: an extracellular, vegetative and a prophage state. Outside the bacterial host the phages are in their extracellular form, which is metabolically inert. Following absorption to the host cell, the nucleic acid penetrates into the interior of the host bacteria and may begin to replicate and give rise to newly synthesised phages called vegetative phages. Phages that exist only in the above two phases are called virulent phages for example *E.coli* T-phages. The latter have been used as a model system for the study of nucleic acid structure and function. Our present understanding on viral DNA replication, transcription and translation of genetic information is based mainly on studies of T-phages.These phages are also used extensively as tools in modern recombinant DNA technology. Phages that are able to enter the prophage state are called temperate phages. When mature temperate phages infect susceptible bacterial cells, these phages may enter either a vegetative state in which they lyse their hosts or they may establish a hereditary symbiotic relationship with the host cells known as lysogeny.

1.4. Lysogeny

Since the early 1920s, naturally occurring strains of *E.coli* were known to permanently carry phage. These phages were capable of causing lysis of other bacterial strains sensitive to the action of the carried phage. However, a fraction of the bacteria infected with a temperate phage does not lyse but instead survives the infection to give rise to clones of bacteria that carry the infecting phage as a prophage. This is the process of lysogenisation and the bacteria are called lysogenic. The subject of lysogeny remained controversial for many years. In 1950, Lwoff and Gutmann reported microscopic observations of a single-cell culture of lysogenic *Bacillus megatherium* which showed clearly that the lysogenic condition could be perpertrated without intervention of phage particles. They followed a cell progeny through nineteen cell divisions, removing one daughter cell from the microculture after each division. All of the transplated cells produced lysogenic colonies. At no time could phage particles be recovered from the fluid portion of the microdrop. In other microdrops, phage particles were recovered, but after a cell had been seen to lyse. This findings allowed Lwoff to describe lysogeny in terms of which the phenomenon is now understood: each bacterium of a lysogenic strain harbours and maintains a non-infective structure, the prophage, which endows the cell with the ability to give rise to infective phage without the intervention of exogenous phage particles. In a small fraction of the population of growing lysogenic bacteria the prophage becomes spontaneously induced and enters a vegetative state which

results in replication of the phage and its release by cell lysis. The discovery by Lwoff *et al.*, (1950) that exposure of lysogenic *Bacillus megatherium* to small doses of ultraviolet (UV) light was followed by the production of bateriophage by almost every cell in the population has proved in a different way that every lysogenic cell contains a prophage. Agents that are able to stimulate the production of phage by lysogenic strains are called inducing agents. Other conditions that may lead to induction are: exposure to mitomycin C (Otsuji *et al.*, 1959), fluoropyrimidine (Marcovich *et al.*, 1963) and shifts in temperature (Lieb, 1966).

1.5. Nature of the prophage

In considering the relation of the prophage to the bacterial cell, two general alternative hypotheses were offered (Jacob *et al.*, 1958): either the prophage somehow adhered to the chromosome or was inserted into the continuity of the chromosome. This question was resolved by Campbell (1962), who put forward a prophage integration model. Campbell assumed that the chromosome of the vegetative lambda (λ) phage is circular. This assumption allowed him to propose that crossing over between the circular λ phage chromosome and the circular *E.coli* chromosome results in the insertion of the entire phage genome into the continuity of the host cell genome. According to this model, at the moment of lysogenisation specific integration sequences of the phage genome and the att λ locus of the bacterial chromosome pair and carry out a single reciprocal cross-over. This results in the insertion of the phage genome as a linear structure in the *E.coli* chromosome. Campbell's predecessors had already found that the DNA of the infecting λ phage particles is linear. Campbell's hypothesis required, therefore, that after the infection of *E.coli* host cells, the linear DNA molecule of the infecting λ phage be circularised to form the hypothesised circular phage DNA. Indication that the λ phage DNA molecule is capable of circularisation was first given by Hershey *et al.*, (1953). These authors found that some of the intact DNA molecules extracted from λ phage particles sediment faster. They proposed that this minor fraction consists of circularised DNA molecules whose compact ring structure causes them to sediment more rapidly than the less compact, linear DNA molecules. The structural details of the terminal regions responsible for circularisation of the linear λ chromosome were worked out by Kaiser and Wu (1963). The site-specific integration of the λ genome into the *E.coli* chromosome has been analysed in detail (Nash, 1981) and follows the Campbell's model. Two proteins are required for λ integration (Kikuchi

and Nash, 1978), the phage encoded recombinase (int) and the host DNA-binding protein called integration host factor (IHF).

Insertion of a phage genome at a specific chromosomal site is not characteristic of all temperate phages. Phage PI lysogenises by forming a stable plasmid that replicates autonomously and segregates during cell division (Sternberg *et al.*, 1981). Another phage, Mu-1, can be inserted at many sites in the chromosome, even in the middle of a host gene, resulting in the inactivation of that gene (Taylor, 1963).

Regardless of the location of the prophage DNA, all temperate phages maintain most of their genes in a repressed state during lysogeny. All of the promoters for genes involved in the lytic growth are stringently controlled so as not to have any detrimental effect on the host. The repressor responsible for this control is one of a number of proteins encoded by the prophage genome. The existence of repressor in the lysogenic cells has a very important consequence: the cells become immune to superinfection by the homologous phage and by members of the same phage family. The repressor encoded by the endogenous prophage genome is just as active on the superinfecting phage DNA as it is on its own DNA and blocks the transcription of the genes necessary for lytic growth.

1.6. Phage-mediated gene transfer

The lysogenic potential of the temperate bacteriophage has several important ramifications. Temperate phage can mediate transfer of bacterial genetic material from one cell to another. This phenomenon was discovered by Lederberg and one of his students (1951) and was called transduction. Two types of transduction can be distinguished: generalised and restricted (specialised). Generalised transduction arises during the vegetative growth of the bacteriophages. Each bacteriophage has a mechanism for packaging its genome into a proteinaceous capsid and some bacteriophages occasionally package fragments of host DNA instead. This aberrant packaging usually occurs randomly with respect to the host sequences that are packed, and any bacterial gene can therefore be transferred. The amount of host DNA that can be transferred is limited by the size of the phage capsid, because DNA packaging stops when the capsid is full. The packaged DNA is contained in a capsid that is fully competent for binding to a second cell. This leads to injection of host DNA and subsequently recombination with the recipient genome. Because the transducing particles with host DNA do not carry any of the genetic information of the bacteriophage, the recipient cell is able to survive this pseudoinfection.

Temperate phages P22 (Zinder and Lederberg, 1951) and P1 (Lennox, 1955) are capable of generalised transduction of genetic markers from donor to recipient cells, and have been used in gene mapping.

The process of specialised transduction depends on the integrated state of the bacteriophage genome. The excision of the bacteriophage genome from the bacterial chromosome occurs by a recombination mechanism that usually results in precise excision (Nash, 1978). However, the recombination mechanism is not always perfect. Occasionally, the excision occurs such that a nearby bacterial gene is included in the excised phage genome. Once this aberrant excision occurs, the bacterial genes become part of the phage genome for all subsequent infections. The novel phage produced in this manner is called a specialised transducing phage. If that particular transducing phage enters into a lysogenic relationship with a second host, the second host will gain bacterial genes from the previous host (Fig.1.2).

Another mechanism of phage-mediated transfer is phage conversion. One example involves the conversion of somatic O antigen of *Salmonella* (Barksdale and Arden, 1976). A wide variety of temperate phages of *Salmonella* can modify the antigenic properties of the somatic O antigens, for example by addition of a D-glucosyl residue. These converting functions are, however, not essential to the phage as mutants defective to them replicate well (Barksdale and Arden, 1976). A second example of lysogenic conversion concerns *Corynebacterium diphtherae*. Toxigenic strains carry the prophage, and nontoxigenic strains can be made toxigenic by lysogenisation. The structural gene for toxin production is located on the phage genome (Barksdale and Arden, 1976).

1.7. Bacteriophages of *Haemophilus influenzae*

The first *H.influenzae* phage (HPI) was isolated by Harm and Rupert (1963), who showed that infection of *H.influenzae* cells by this phage could be achieved in 3 ways: by allowing whole phage to attach and inject its DNA and by exposing competent *H.influenzae* cells to phage DNA or to DNA extracted from lysogenic cells. A year later *H.influenzae* phage S2 was recovered from a patient at the University of Pennsylvania Hospital (Goodgal, 1964). Phages HPI and S2 are morphologically and genetically similar. They have an icosahedral head and a complex tail structure. The duplex DNA of mature HPI and S2 phages shows about 90% homology as judged by hybridisation (Boling *et al.*, 1973; Fitzmaurice *et al.*, 1984). It has been reported that both HPI and S2 phages have unique but different

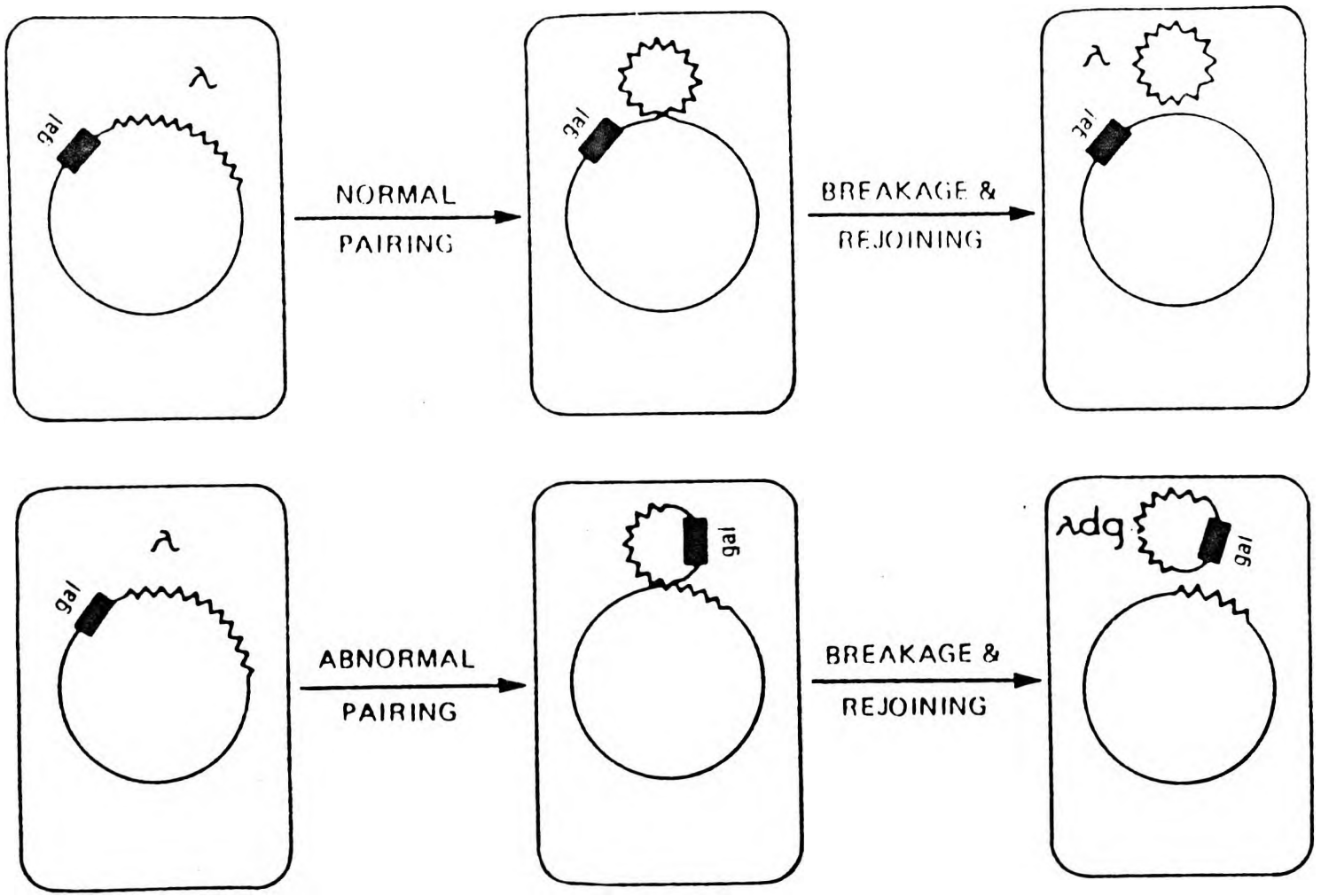


Figure 1.2 Upper row : Detachment of λ prophage to form a normal λ vegetative DNA.
Lower row : Detachment of λ prophage to form λ defective carring gal genes DNA, which will mature as a transducing particle .

sites of integration on the chromosome of *H.influenzae* (Stuy, 1985). Physical maps constructed by localisation of the cleavage site of several restriction endonucleases have shown that each of the *Haemophilus* bacteriophages (S2 and HPI) exists in 3 forms which differ in the molecular organisation of the genome (Piekarowicz *et al.*, 1986). This led to the conclusion that some differences between S2 and HPIcl phages such as different immunity, 10% non-homology (Boling *et al.*, 1973) and different insertion sites (Stuy, 1985) were in fact related to the molecular organisation of the S2 and HPIcl phages. The hosts for HPI and S2 are all rough segregants of various serological types of *Haemophilus influenzae* (Piekarowicz and Glover, 1972; Stuy,1978). In the lysogenic state, HPIcl genome is inserted into *H.influenzae* chromosome at a single locus (Waldmann *et a*l.,1986), as a consequence of recombination between specific phage and chromosomal sites. The HPIcl insertion is distinguished by the large size core, which is common to the phage and chromosomal regions. This core contains 182 residues and consists of two blocks of 93 and 62 residues identical in both phage and host site which are separated by a 27-base-pair (bp) containing 6 mismatched residues. Recombination occurs in the 62 bp block (Waldman *et al.*,1986). The genome structure of HPI lysogens resembles that of λ and other temperate coliphages in that it has cohesive ends (Boling *et al.*, 1973; Fitzmaurice *et al.*, 1984) making it likely that insertion of the HPI genome into the cognate host site results from a similar recombination process, and follows Campbell's model (Campbell, 1950). As is the case in λ integration, the phage encoded recombination protein int and the host DNA binding protein IHF are important in the integration of phage HPIcl (Goodman and Scocca, 1989).

By ultraviolet irradiation of 20 strains of *H.influenzae* isolated from clinical specimens, Samuels and Clarke (1969) recovered three phages of which one, (N3) could be distinguished from phages HPI and S2. Phage N3 formed plaques on only 1 of the 20 strains tested. DNA from phage N3 could be successfully used for transfection of different *H.influenzae* strains (Samuels and Clarke, 1969). *H.influenzae* bacteriophage N3 differs from *Haemophilus* phages HPI and S2 morphologically. It has a noncontractile tail, and a hexagonal head (Samuels and Clarke, 1969). The double stranded DNA of N3 has cohesive ends (Filipska *et al.*, 1981). Phage N3 can code for its own recombination system (Jablonska *et al.*, 1976), while HPI and S2 make use of the host cell enzymes for vegetative recombination (Boling and Setlow, 1969). One important feature of N3 bacteriophage is that it inhibits transformation and transfection (Piekarowicz *et al.*, 1977). This inhibition

was due partly to the decreased capacity of competent lysogenic cells for irreversible binding of DNA and partly to some events taking place after absorption of DNA (Piekarowiz *et al.*, 1977).

The first defective *H.influenzae* phage was recovered from a highly transformable *H.influenzae* strain (Rd). This strain was able to undergo lysis after UV irradiation (Stachura *et al.*, 1969). However, the lysate was not able to produce plaques when tested on other *H.influenzae* strains. Because of the inability to find a suitable host it was assumed that the phage from strain Rd was defective. This phage was morphologically similar to HPIcl and S2. The phage exhibited an altered tail plate-fiber assembly (Boling *et al.*, 1973), which could possibly account for the lack of attachment of this phage to the baterial cell. The lack of cohesive ends in the phage DNA (Boling *et al.*, 1973) may be another reason why no host was found for this phage. In crude lysates this phage appeared to have empty heads and presumably did not contain DNA (Stachura *et al.*, 1969). Purification of the phage from strain Rd resulted in virus particles that contained DNA (Boling *et al.*, 1973). The phage DNA showed little or no homology to DNA from the other two phages HPI and S2 although it was approximately the same size. Several defective phage genetically related to HPI and HP3 were reported by Stuy (1978). These phages were recovered from nontypable *H.influenzae* isolates and one defective phage was isolated from a serotype b isolate of *H. influenzae*. Phage HP3 was recovered from a nontypable *H.influenzae* strain (Stuy, 1978). This phage as well as the defective phages reported by Stuy have not been characterised morphologically.

1.8. Transfection in *Haemophilus influenzae*

Transfection is infection of cells by nucleic acid extracted from a phage which results in virus production. Transfection of *H.influenzae* competent cells have been reported by Harm and Rupert (1963) and Samuels and Clarke (1969). DNA from phage HPIcl transfects both rec+ and rec- recipients (Stuy, 1986): transfection thus does not require any recombination event as was reported by Notani *et al.*, (1976). The intracellular events during infection by *H.influenzae* phage DNA have been described by Stuy in 1986. According to the transformosome hypothesis (Kahn and Smith, 1984), phage DNA molecules, like other adsorbed double-stranded DNA molecules, are released from the transformasomes as broken single stranded molecules. A small fraction of the adsorbed phage DNA duplex molecules is not normally processed into single strands upon release from the transformasome.

Instead, they escape this process. The efficiency of phage DNA transfection thus depends on the number of intact DNA duplex molecules.

1.9. Phage restriction and modification in *Haemophilus influenzae*

In 1973 Wilcox and Smith discovered the first restriction enzyme in *H.influenzae* Rd (Hind III). The characteristic feature of this enzyme is its ability to produce specific breaks on heterologous DNA, while homologous DNA was protected from the restriction enzyme. This is due to the presence of a modification enzyme which is able to methylate the restriction sites. This important discovery is considered as the beginning of the era of recombinant DNA technology. The genetically related phage HPI and S2 were used for studying the properties of phage restriction and modification systems in *H.influenzae* (Gromkova *et al.*, 1973; Setlow *et al.*, 1968). Rough variants of different *H.influenzae* serotypes have different restriction phenotypes due to the presence of different class 1 restriction enzymes. Each restriction enzyme is paired with a corresponding site-specific DNA modification enzyme. Ra , Re and Rb11 strains each have two different modification enzymes (Piekarowicz and Glover, 1972; Piekarowicz, Kalinowska, 1974) whereas Rd and Rb1 strains each have one modification enzyme (Glover and Piekarowicz, 1972; Gromkova *et al*, 1973; Piekarowicz *et al.*, 1974). Rc strains have no such enzyme, whereas Rf strains may have more than one.

Some of the genes involved in phage modification and restriction have been located and the phage restriction enzyme isolated (Gromkova *et al.*, 1974). These genes are located between bio + and pro + in the *H.influenzae* chromosome. This restriction enzyme inactivates the transfection activity of nonmodified phage DNA but does not attack modified phage DNA.

1.10 Events leading up to the studies comprising this dissertation

Encapsulated strains of *Haemophilus influenzae* have the ability to undergo autolysis (Riley, 1981; Alexander and Leidy, 1943). The mechanism of this activity is not clear. Some authors believe that the autolysis is due to the presence of bacteriophages. However, there are only a few published reports on the recovery of lysogenic strains of *H.influenzae* from clinical specimens (Samuels and Clarke, 1969; Stuy, 1978) and no information is available on the effect of serotype b capsule of this organism on cell lysis and the ability of *H.influenzae* cells to become lysogenised. In 1969 Samuels and Clarke reported that 3 out of 20 clinical isolates of

H.influenzae were able to produce phage after ultraviolet irradiation but the serotype of the strains tested was not indicated (Samuels and Clarke, 1969). Only 1 out of 92 encapsulated strains of *H.influenzae* was found by Stuy (1978) to be lysogenic to a defective phage related to the HP3 phage of *H.influenzae* while 17 out of 71 nontypable isolates were lysogenic for either HPI or HP3 defective phages. The author concluded that *H.influenzae* capsules reduce greatly the ability of a given cell to become lysogenised. In Stuy's investigation, only spontaneously produced bacteriophages had been studied. No attempt were made to induce lysis by some of the commonly used inducing agents such as mitomycin C or UV. Furthermore, a limited number of indicator strains were used. There is only one published report on the transmission of both chromosomal and plasmid genes by bacteriophage in *Haemophilus* species (Gromkova and Goodgal, 1977). The transfer of the gene conferring ampicillin resistance was mediated by a defective *H.parainfluenzae* bacteriophage. With the discovery of new phages it may become possible to develop a typing system which could be used in epidemiological and diagnostic studies.

2. CHARACTERISATION OF BACTERIAL STRAINS EXAMINED FOR LYSOGENY

2.1. Introduction

Haemophilus influenzae belongs to the genus *Haemophilus*. The term *Haemophilus* is derived from the requirement of factors found in blood for growth, i.e. (Greek for blood) and philus (Greek for loving). *Haemophilus influenzae* was first isolated by Pfeiffer during the 1892 influenza pandemic. The frequency of its presence in the nasopharynx of influenza patients and post mortem lung cultures led to the erroneous assumption that it was the aetiological agent of influenza, thus the designation "the influenza bacillus". As was later shown, influenza is caused by a virus.

H.influenzae is a Gram-negative rod. A characteristic morphological property of *H.influenzae* is its pleomorphism. Although predominatly a small coccobacillus, it often grows in short chains or in long filaments.

Man is the natural host of *H.influenzae* and it is usually present in the nasopharynx of healthy individuals. *H.influenzae* is a major cause of acute bacterial meningitis in infancy and early childhood. It is also the cause of acute epiglottis, cellulitis and pyarthrosis (Riley, 1981).

Pittman (1931) discovered that the strains of *H.influenzae* that cause meningitis and other acute infections differ from those found in the respiratory tract of healthy individuals in that they possess capsules. She established that the encapsulated strains may be divided into six capsular serotypes designated a through f . Of the six capsular serotypes demonstrated by Pittman, serotype b organisms are responsible for most acute infections caused by *H.influenzae* (Riley, 1981). There is also an increasing awareness of the pathogenic potential of non-typable *H.influenzae* strains among adults where they are commonly associated with chronic pulmonary diseases. Recent reports indicate that nontypable *H.influenzae* can cause acute infections in children (Riley, 1981).

The laboratory identification and classification of species of the genus *Haemophilus* are based on their requirements for either NAD (V-factor) or haemin (X-factor) or both. *H.influenzae* require both factors for its growth.Chocolate agar is the most generally used culture medium for the isolation of *Haemophilus influenzae*. In its preparation, blood is added to an agar base and heated at 80°C until a brown colour

appears. The mild heat releases the X and V factors from the blood cells and also inactivates V factor splitting enzymes without destroying the V factor itself. Also useful for culturing of clinical specimens is Levinthal and Fildes enriched agar medium. Ordinary blood agar permits growth of *H.influenzae* only when the agar plate is cross-inoculated with a *Staphyloccusus* species or other V factor-excreting microorganisms. Sizable colonies appear in the vicinity of the feeder strain. This phenomenon is known as satellitism.

In the scheme proposed by Kilian (1976), *H.influenzae* could be divided into several biotypes based on indole production, urease activity, and ornithine decarboxylase. To date 6 biotypes of *Haemophilus influenzae* have been identified. Specific biotypes have been associated with different types of infections, source of isolation (Abritton *et al.*, 1978; Braun and Frus-Moller, 1976; Controni *et al.*, 1981; Frederikson, 1979; Kilian *et al.*, 1979; Wallace *et al.*, 1983) and antimicrobial susceptibility pattern (Albritton *et al.*, 1978).

2.2. Materials and methods

2.2.1. Bacterial strains

A total of 58 clinical isolates of *Haemophilus influenzae* were tested for lysogeny. Twenty-seven strains were recovered from paediatric patients at Baragwanath Hospital (Table 2.1). Fifteen of these strains were from patients with meningitis, 8 strains were from patients with pharyngitis and 4 strains were recovered from bacteraemic patients. Thirty-one strains were isolated from respiratory secretions of healthy children at a creche in Johannesburg and from paediatric patients at Baragwanath Hospital.

2.2.2. Media

H.influenzae strains were grown in brain heart infusion (BHI) broth and on BHI agar from Becton Dickenson Co. (Cockeysville, Maryland) supplemented with 10ug/ml haemin (X factor) and 2μg/ml nicotinamide dinucleotide (NAD) both from Sigma Chemicals Co., St Louis, Missouri.

2.2.3. Identification

All strains received were confirmed as *Haemophilus influenzae* by testing their ability to grow on the following media: BHI; BHI + NAD; and BHI + NAD

+ Haemin. Strains which grew only on BHI plate containing Haemin and NAD were identified as *H.influenzae*.

Further identification was made by a porphyrin test. The substrate consisted of 2mM S-aminolevulinic acid-hydrochloride (Sigma Chemical Corporation) and 0.8mM MgS04 in 0.1M phosphate buffer at pH 6.9 and distributed in 0.5ml quantities in small tubes. A heavy loopful of bacterial culture from BHI agar was resuspended in the substrate, and incubated overnight at 37°C. After incubation, the substrate was exposed to UV light. A red fluorescence from the baterial pellet or from the fluid was considered positive for porphyrin. *Haemophilus parainfluenzae* was included as a positive control.

2.2.4. Serotyping

Capsular serotypes were determined by slide agglutination with six (a through f) type specific antisera (Wellcome Diagnostics). A sample of an 18-hour culture grown on BHI agar plates was emulsified in a drop of saline to give a smooth, fairly dense suspension on a glass microscope slide. A drop of undiluted antisera was added and then mixed. The slide was rocked gently observing for agglutination and results were read within a minute. A drop of saline and bacterial culture was included as a control to exclude auto-agglutination.

The presence of capsules was further confirmed by demonstrating colony irridescence. Logarithimically grown broth culture was plated on supplemented BHI agar to yield isolated colonies. The plates were viewed under a stereomicroscope with obliquely transmitted light for colony iresdescence.

2.2.5. Isolation of nonencapsulated variants.

Nonencapsulated variants were isolated by several passages in supplemented BHI broth followed by plating on BHI agar. The colonies were tested for agglutination and for iridescence. (see above). Only colonies that had lost both iridescence and failed to agglutinate were considered to be true nonencapsulated variants.

2.2.6. Biotype determination

Strains were assigned to one of 6 biotypes on the basis of indole, urease and ornithine decarboxylase production (Kilian, 1976).

2.2.6.1. Indole test

The substrate used was 0.1% L-tryptophan in 0.05M phosphate buffer at pH 6.8. The substrate was distributed in glass test tubes. A heavy inoculum of pure culture was suspended in the substrate, and tubes were incubated overnight. Few drops of Kovacs reagent were added to the tubes and mixed.

2.2.6.2. Urease test

The Christensen's urea agar medium was used (Koneman *et al.*, 1983). The surface of the agar slant was streaked with the test organism and incubated at 35°C for 18 hours.

2.2.6.3. Ornithine decarboxylase

Moeller decarboxylase medium (Koneman *et al.*, 1983) containing ornithine amino acid was used. A heavy inoculum of the test organism previously recovered on supplemented BHI or chocolate agar plates was inoculated onto the Moeller decarboxylase medium. The tubes were overlaid with mineral oil and incubated at 35°C for 18 to 24 hours.

2.2.7. Antibiotic resistance testing

The strains were tested for susceptibility to ampicillin, tetracyline and chloramphenicol by the disc method. The nitrocephin method was used to demonstrate the elaboration of beta-lactamase (O'Calloghan *et al.*, 1972).

2.2.8. Storage of cultures

Strains were stored by freezing logarithmically grown broth cultures in 20% glycerol at -70°C.

2.3. Results

All strains isolated from healthy children and 3 strains recovered from patients were nontypable while 24 strains isolated from patients belonged to encapsulated serotype b (Table 2.1). *H.influenzae* serotype b strains agglutinated strongly with type b antisera and did not agglutinate with the remaining five type-specific antisera. They all produced opaque and iridescent colonies, characteristic of encapsulated *H.influenzae*. Nontypable strains failed to agglutinate any of the six type-specific antisera and were not irisdescent when examined by stereomicroscope with obliquely transmitted light. The characteristics of *H.influenzae* clinical isolates recovered from patients that were tested for lysogeny are presented in Table 2.1. Twelve *H.influenzae* serotype b strains were found to be biotype I, eleven biotype II and one biotype III. Ten nontypable *H.influenzae* isotypes were biotype I, eleven biotype II, 9 biotype III and two each from biotype IV and V.

Table 2.1 Characteristics of *Haemophilus influenzae* clinical isolates recovered from patients.

STRAIN	SPECIMEN	SEROTYPE	BIOTYPE
35910	CSF	b	I
37710	CSF	b	II
122155	CSF	b	I
126814	CSF	b	III
14945	CSF	b	II
12226	Blood	b	II
122160	CSF	b	II
15831	CSF	b	II
14981	CSF	b	I
2457	CSF	b	I
122178	CSF	b	II
59797	Blood	b	II
01541	Blood	b	I
12	TS[b]	b	I
168	TS	b	II
5	TS	b	I
11	TS	b	I
13	TS	b	II
64	TS	b	I
451	TS	b	I
77077	CSF	b	II
71781	CSF	b	I
10674	CSF	b	II
1	TS	b	II
7515	CSF	NT[c]	IV
122165	CSF	NT	II
02523	Blood	NT	IV

[a] CSF = cerebrospinal fluid

[b] TS = throat swab

[c] NT = nontypable

3. PROPHAGE INDUCTION AND PROPERTIES OF LYSOGENIC STRAINS OF *HAEMOPHILUS INFLUENZAE*

3.1. Introduction

The termination of the lysogenic state, or prophage induction requires both release from repression and excision of phage DNA from the host chromosome. Induction is usually followed by synthesis of the phage components, formation of mature phage particles and their release by cell lysis. Release of phage may occur spontaneously at a low frequency, but occurs in nearly every lysogenic cell after treatment with various physical and chemical agents called inducing agents, such as ultraviolet (UV) light, mitomycin C, nitrogen mustard, hydrogen peroxide and organic peroxide. In addition, thymine starvation of thymine auxotrophs and temperature elevation in certain mutants unable to synthesize phage DNA can act as inducing procedures. What all these agents/procedures share in common is that they arrest cellular DNA replication.

It has been shown in *Escherichia coli* K12 that a variety of conditions that damage DNA or block host DNA replication activate the protease activity of the host rec A protein to induce the SOS pathway (Friedberg, 1985; Witkin, 1976). The Rec A protease in addition to cleaving the lex A repressor, also cleaves the λ repressor. This results in the release of λ genes from repressed state and the occurrence of cell lysis. Although the mechanism of induction in *H.influenzae* is not clear, it is reasonable to assume that the SOS mechanism also plays a role in the induction of S2 and HPIcl, since rec 1 mutants, which have many of the characteristics of *E.coli* recA mutants (Kooistra and Setlow, 1976) were not inducible by UV and mitomycin C (Balganesh and Setlow, 1984; George and Notani, 1980). Regardless of possessing its own recombination system, N3 requires the host rec 1 function for induction (Jablonska *et al.*, 1976). Rec 1 protein is not required for ethyl methanosulfonate (EMS) induction of HPIcl prophage since the rec 1 mutant could be induced to a significant level by EMS (Balganesh and Setlow, 1984). There apparently exist both Rec + dependent and independent mechanisms of phage induction in *H.influenzae*.

The objectives of the experiments presented in this chapter were:

- to investigate how frequently lysogenic strains occur among encapsulated and nontypable clinical isolates of *H.influenzae*;

- to study the effect of serotype b capsule on both cell lysis and the ability of cells to become lysogenised under natural conditions. In addition, some of the properties of the lysogenic strains were investigated.

3.2. Materials and Methods

3.2.1. Bacterial strains used

A total of 58 isolates of *H.influenzae* were tested for their ability to undergo lysis spontaneously and after treatment with mitomycin C and UV light.

3.2.2. Spontaneous lysis

Overnight BHI cultures of *H.influenzae* isolates were diluted 1 in 10 in supplemented BHI broth and grown at 37°C with shaking. Samples were taken after 5, 7 and 24 hours incubation for determination of optical density (OD) at 650nm, and the concentration of viable bacteria based on the number of colony forming units (CFU).

3.2.3. Mitomycin C treatment

Overnight BHI cultures diluted as above were grown by shaking in supplemented BHI broth at 37°C to an optical density of 0.1 at 650nm. Mitomycin C (Sigma Chemical Corporation, St. Louis, Missouri) was added to a final concentration of 0.05μg/ml and the culture incubated further. Samples were taken at 30 min intervals for determination of OD and CFU.

3.2.4. Effect of chloramphenicol on prophage induction

Chloramphenicol at a concentration of 100μg/ml was added immediately after the addition of mitomycin C (see section 3.2.3). After 30 min incubation at 37°C the cells were centrifuged at 8000 rpm for 10 min, washed once with BHI broth and resuspended in a supplemented BHI broth to the initial volume. The culture was incubated at 37°C with shaking. Samples were taken at 30 minute intervals for determination of OD.

3.2.5. Ultraviolet (UV) light induction

Ten millilitre supplemented BHI broth was inoculated with 0.2ml of an overnight BHI culture of *H.influenzae* and aerated at 37°C to OD of 0.5 at 650nm. The culture was placed in ice for 10 minutes and the cells were

pelleted by centrifugation at 8000 rpm for 10 minutes. The pellet was resuspended in saline to give approximately 10^9 CFU/ml. Five ml samples were then irradiated with UV light in 9cm petri-dishes under a Toshiba 15-W germicidal lamp for 5 minutes (about 10% survival). After irradiation, cells were diluted five fold in fresh supplemented BHI broth and 10ml samples were transferred to a 100ml Erlenmeyer flask. Incubation with shaking was continued at 37°C and OD was determined at 30 minutes intervals.

3.3. Results and Discussion

3.3.1. Prophage induction by mitomycin C

Nine clinical strains of encapsulated *H.influenzae* were able to undergo lysis after treatment with mitomycin C (Table 3.1). Fig. 3.1 shows the kinetics of cell lysis of a lysogenic encapsulated *H.influenzae* type b clinical isolate (strain 15831) grown in the presence or absence of 0.05μg/ml mitomycin C. The optical density of the induced culture increased for about 2½ hours after the addition of mitomycin C, followed by a gradual decrease during the next few hours. OD readings were not markedly reduced probably due to the presence of cell debris. The induced bacteria began to lose their colony forming ability soon after addition of mitomycin C. The untreated culture did not undergo lysis and its growth followed the profile of a normal growth curve. The remaining *H.influenzae* lysogenic strains that possessed type b capsule showed a similar profile of cell lysis and CFU to that of strain 15831 after treatment with mitomycin C. Nontypable *H.influenzae* clinical isolates were also investigated for their ability to undergo lysis after treatment with mitomycin C. Seven nontypable strains were able to undergo lysis (Table 3.1). Fig. 3.2 shows the kinetics of cell lysis of a lysogenic *H.influenzae* nontypable strain 45B. The lytic curve of this strain is similar to that of encapsulated strain 15831 described above.

Table 3.1 Inducible strains of *H.influenzae* recovered from healthy children and patients.

H. INFLUENZAE STRAIN NO.	SOURCE	SPECIMEN	SEROTYPE	BIOTYPE
45B	Healthy	TS[a]	NT[b]	V
35B	Healthy	TS	NT	I
14W	Healthy	TS	NT	V
18W	Healthy	TS	NT	I
168	Patient	CSF[c]	b	I
53	Healthy	TS	NT	II
122165	Patient	CFS	NT	II
59	Healthy	TS	NT	II
15831	Patient	CSF	b	II
122160	Patient	CFS	b	II
35910	Patient	CSF	b	I
14981	Patient	CSF	b	I
14945	Patient	TS	b	II
1	Patient	TS	b	II
11	Patient	TS	b	I
13	Patient	TS	b	II

[a] TS = throat swab

[b] NT = nontypable

[c] CSF = cerebrospinal fluid

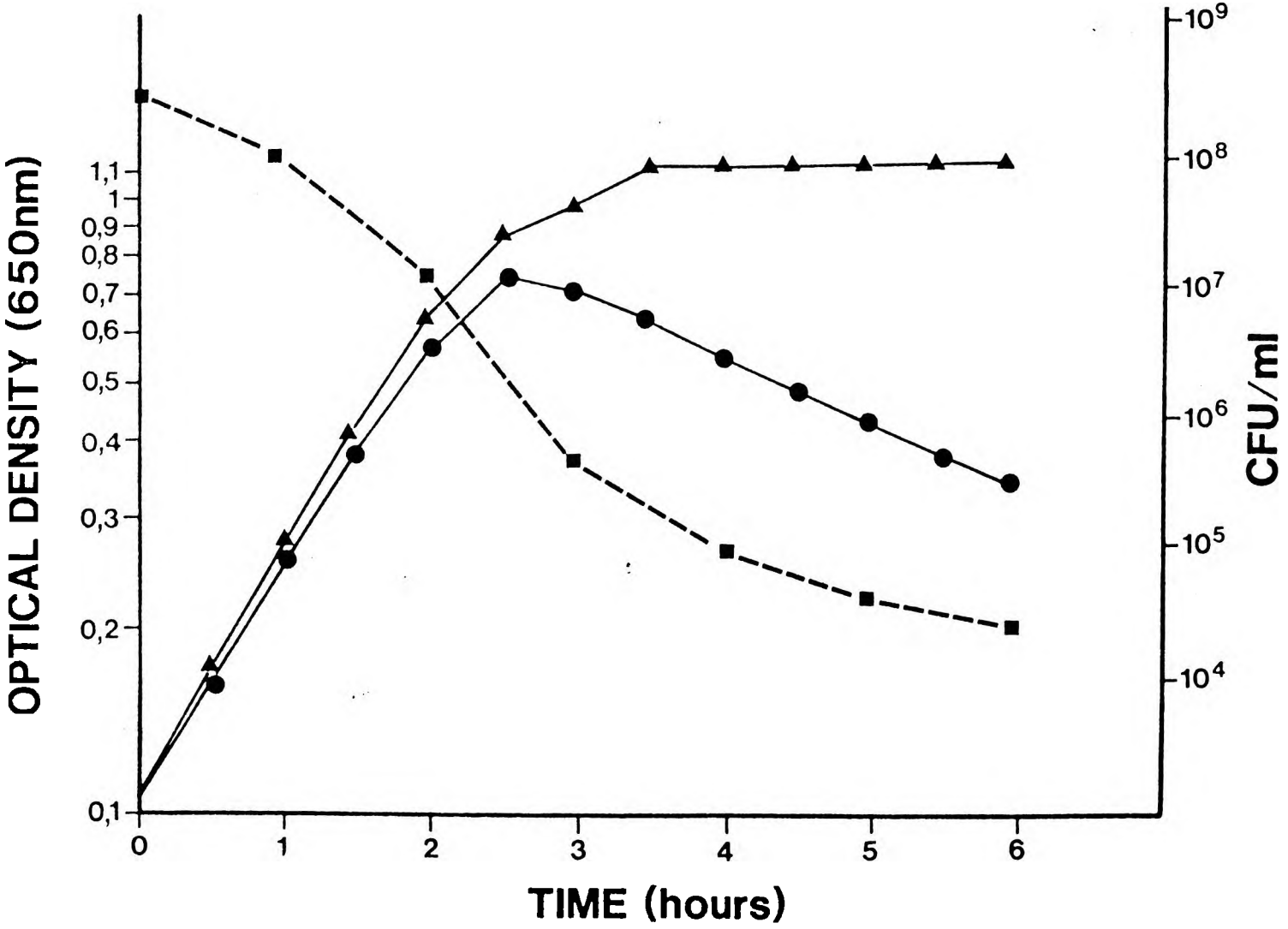


Figure 3.1. Kinetics of cell lysis of a lysogenic encapsulated *H. influenzae* type b clinical isolate (strain 15831). Symbols : (●) culture treated with mitomycin C; (▲) culture untreated with mitomycin C; (■) colony forming unit of culture treated with mitomycin C.

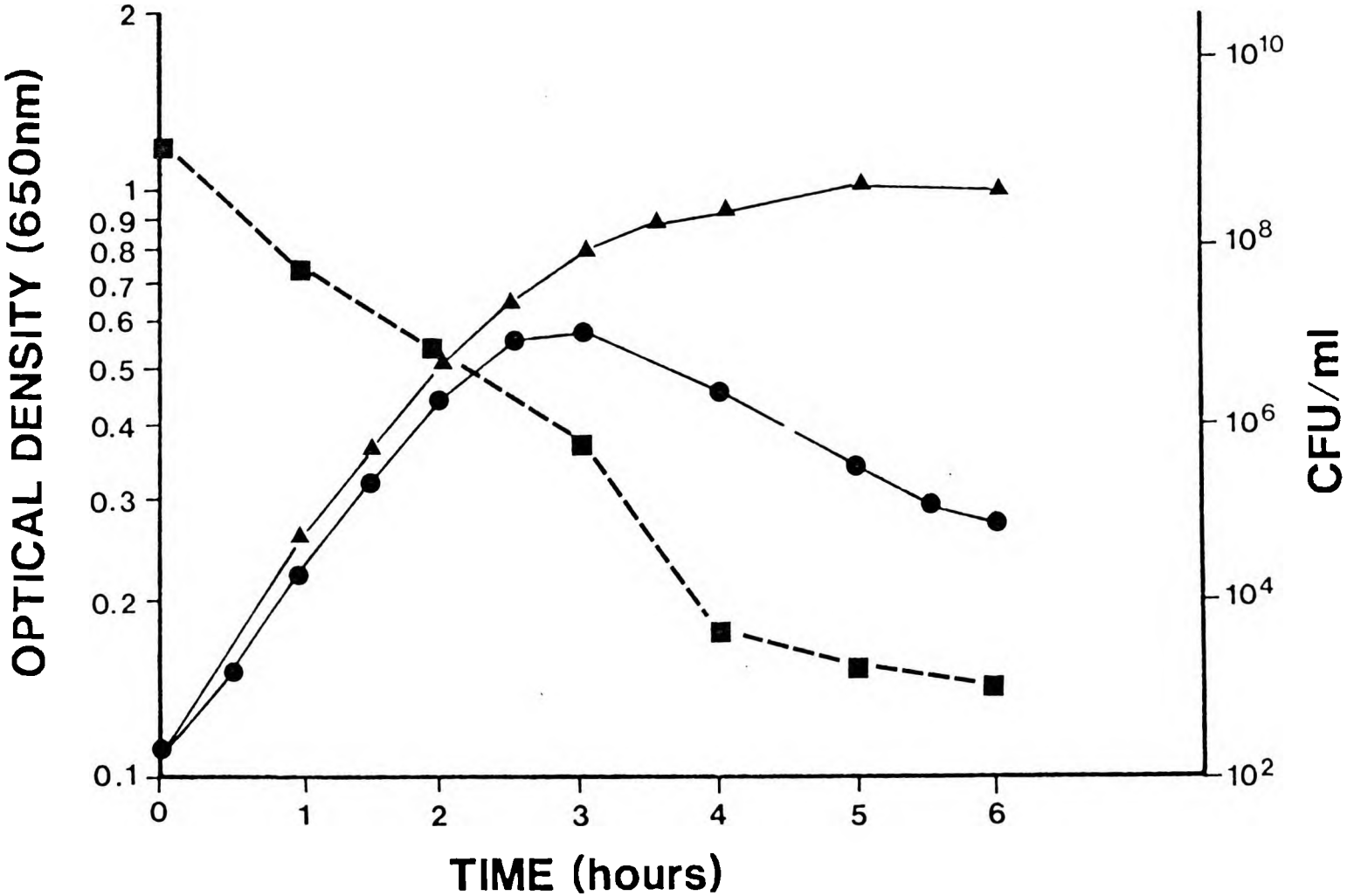


Figure 3.2. Kinetics of cell lysis of a lysogenic nontypable *H. influenzae* strain 45.
Symbols : (●) culture treated with mitomycin C; (▲) culture untreated with mitomycin C; (■) colony forming unit of culture treated with mitomycin C.

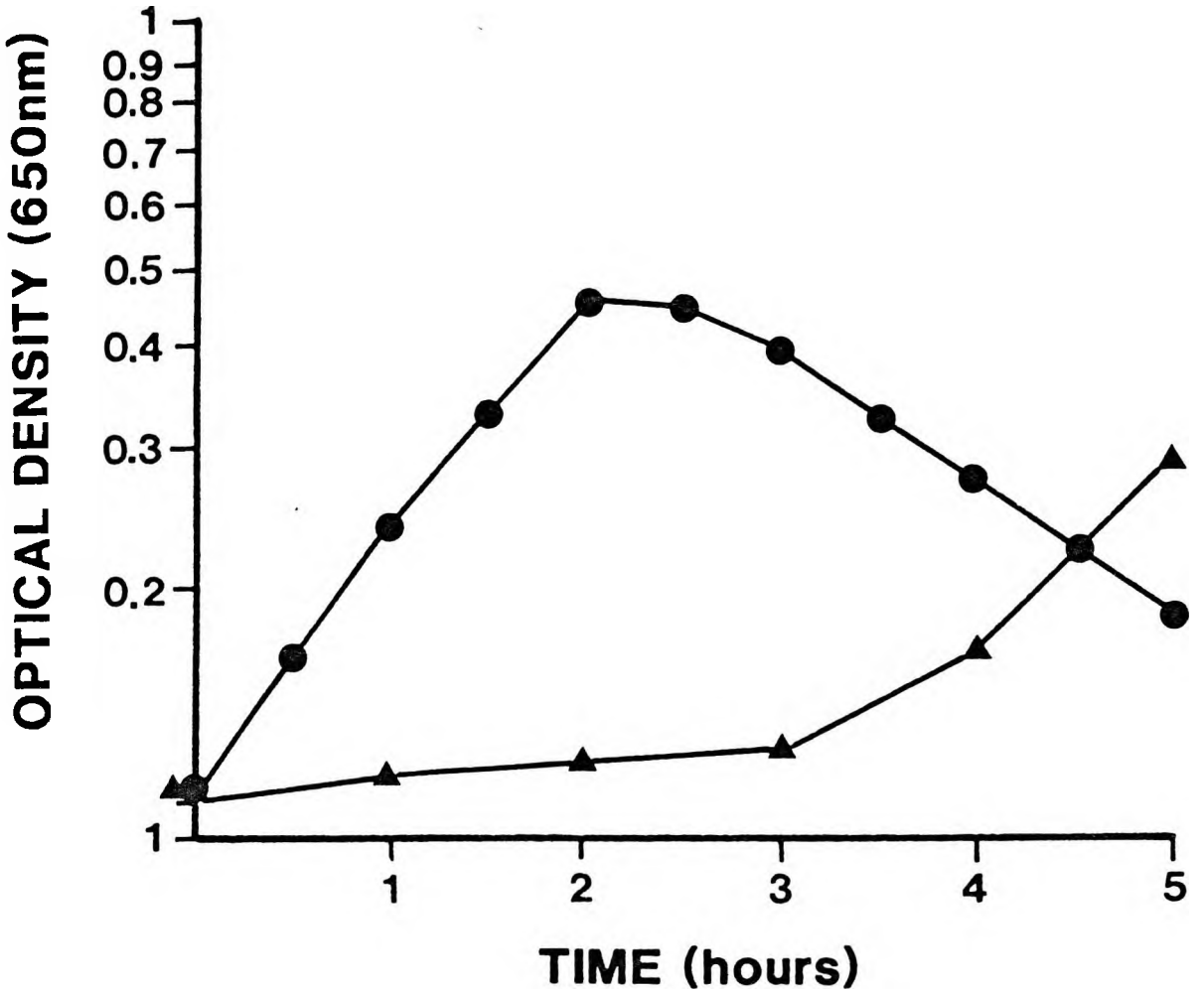


Figure 3.3 Kinetics of cell lysis of a lysogenic strain 168 induced with mitomycin C in the presence (▲) and absence (●) of 100μg/ml chloramphenicol.

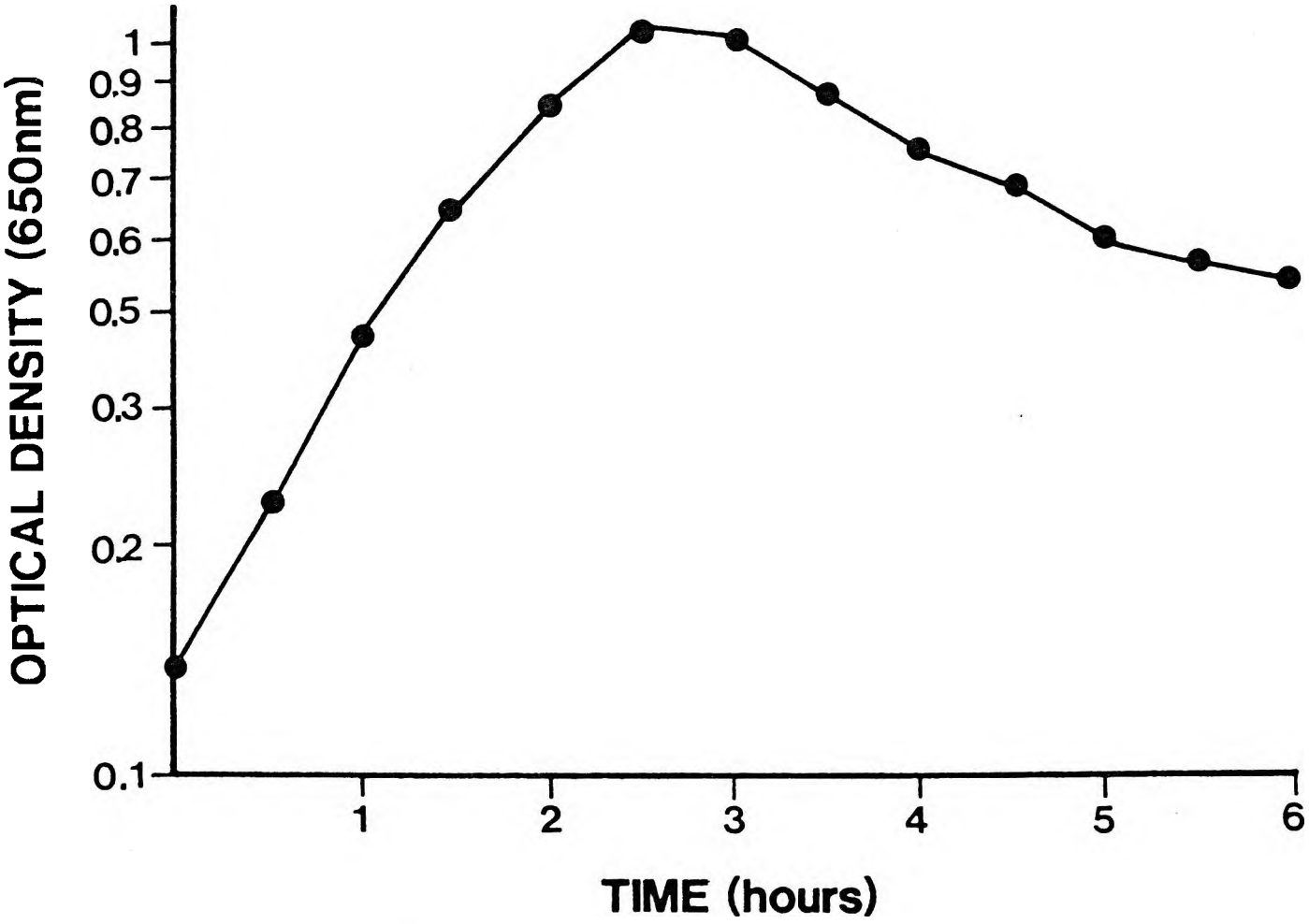


Figure 3.4 Kinetics of cell lysis of strain 15831 after induction with UV light.

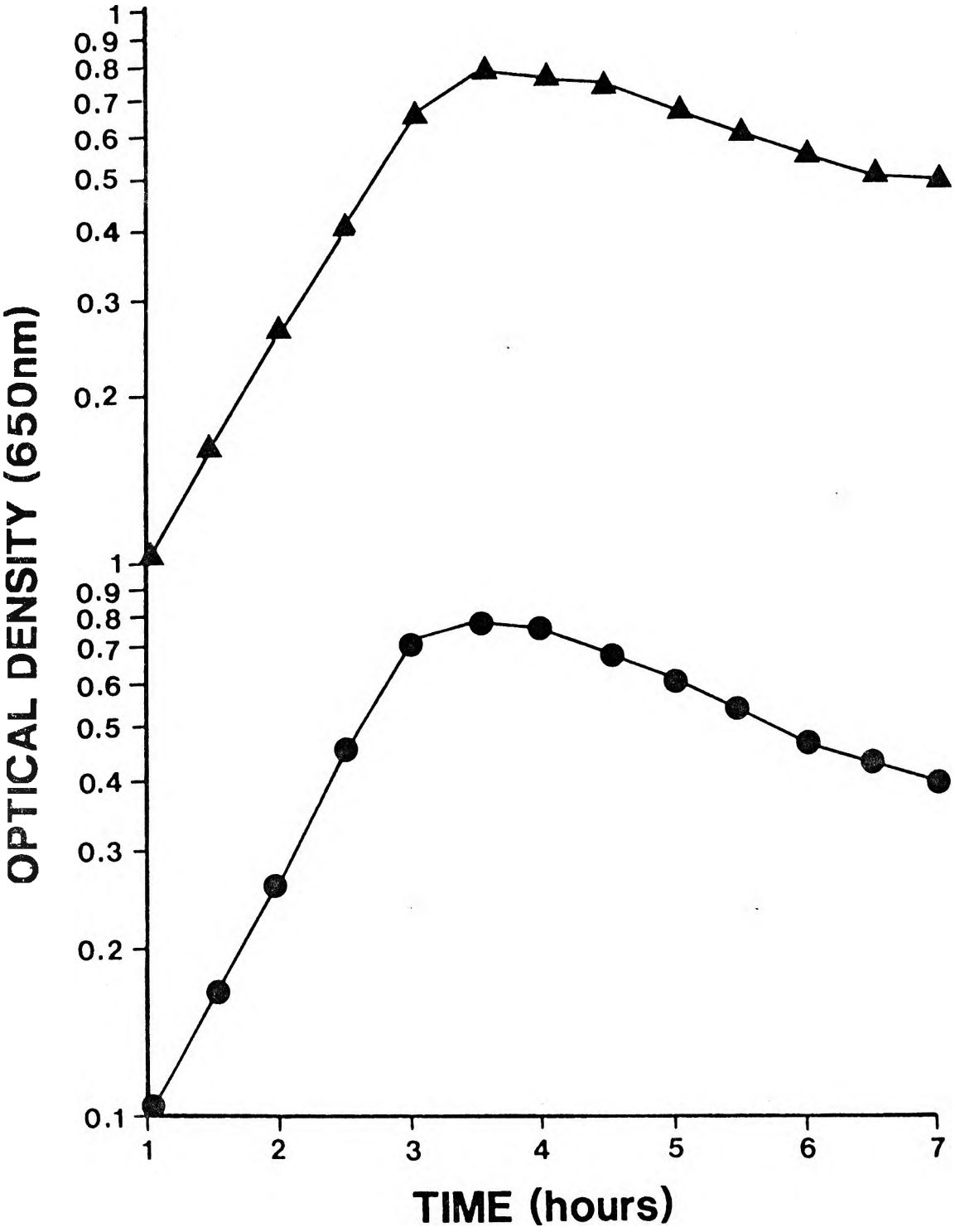


Figure 3.5 Kinetics of cell lysis of lysogenic encapsulated strain (15831S) and its corresponding nonencapsulated varient (15831R). Symbols : (●) encapsulated; (▲) nonencapsulated.

3.3.2. Effect of chloramphenicol on prophage induction by mitomycin C

Addition of chloramphenicol suppressed the lysis of all 16 *H.influenzae* isolates that were inducible by mitomycin C. Fig 3.3 shows the kinetics of cell lysis of a lysogenic strain 168 induced with mitomycin C in the presence and absence of 100μg/ml chloramphenicol. The rise in the turbidity of the culture during the first 2 ½ hours after addition of mitomycin C (Figures 3.1 and 3.2) indicates that cell division and DNA replication continued during induction. It can be seen that the increase in optical density was prevented by the addition of chloramphenicol which is known to inhibit protein synthesis. The inhibition of lysis by chloramphenicol suggests that synthesis of new proteins was essential for the induction process. Mitomycin C itself was therefore not directly responsible for cell lysis. Protein synthesis is also required for the inactivation of the repressor in C600 (λ) (Shinagawa and Itoh, 1973) and in *Bacillus subtilis* PBSX phage (Seaman *et al.*, 1964) after induction by mitomycin C.

3.3.3. Prophage induction by UV light

Sixteen *H.influenzae* isolates which were able to undergo lysis after mitomycin C treatment, were found to lyse after UV irradiation. Fig 3.4 shows the kinetics of cell lysis of strain 15831 after induction with UV light. As in the case of mitomycin C induction the OD of the induced culture increased for about 2½ hours after treatment with UV light, followed by a gradual decrease during the next few hours.

3.3.3.1. Spontaneous lysis

Studies on the ability of *H.influenzae* clinical isolates to undergo lysis spontaneously showed lack of decrease in OD in most isolates when grown in absence of inducing agents. Only few strains showed slight decrease in OD after 18 hours incubation at 37^{o}C when compared with that of a 7-hour-incubated culture. However, when the viable counts of both mitomycin C-inducible and mitomycin C-noninducible cultures were compared (Table 3.2) it was found that the viable counts of both groups of bacteria were greatly reduced after 18 hours incubation (about 100 to 1000 fold reduction). These data suggest that the significant loss in viability of *H.influenzae* after overnight incubation

Table 3.2. Spontaneous lysis of *H.influenzae* strains.

		7 -HOUR CULTURE		24-HOUR CULTURE	
STRAIN	MITOMYCIN C INDUCTION	OD	CFU	OD	CFU
15831	+[a]	1.189	$1.3X10^{10}$	0.916	$5X10^{7I}$
71781	-[a]	1.062	$1.5x10^{10}$	1.067	$1.4x10^{7}$
13	+	1.060	$.9x10^{9}$	1.107	$7.23x10^{7}$
34B	+	1.432	$1.2x10^{10}$	1.251	$2.2x10^{6}$
12B	-	1.262	$2.1x10^{10}$	1.285	$1.23x10^{7}$
122165	+	1.235	$1.8x10^{10}$	1.012	$4.08x10^{7}$
Rd	+	1.356	$4.7x10^{9}$	1.255	$9.31x10^{7}$

[a] + inducible by mitomycin C,

[a]- noninducible by mitomycin C

was not due to the spontaneous production of phage by the lysogenic strains. However, no attempt were made to determine the amount of cell debris present.

3.3.4. Effect of type b capsule on prophage induction and cell lysis

In order to study the effect of type b capsule on prophage induction and cell lysis, nonencapsulated variants were selected from 2 lysogenic encapsulated *H.influenzae* type b strains (15831 and 35910) that were able to undergo lysis after treatment with mitomycin C and 6 encapsulated type b (strains 5, 122153, 2457, 126814, 12 and 122178) that were not inducible by this agent. Fig.3.5 shows the kinetics of cell lysis of lysogenic encapsulated strain (15831S) and its corresponding nonencapsulated variant (15831R). The lytic curves of the encapsulated strains were indistinguishable from those of their corresponding nonencapsulated variants. The presence of capsule during the lytic period was confirmed by slide agglutination with serotype b antiserum while the remaining type-specific antisera did not agglutinate the culture. Studies of the effect of the capsule on cell lysis in other bacterial systems such as *Streptococcus pneumoniae* (Bernheimer and Tiraby, 1976) have provided evidence that as is the case with *H.influenzae,* capsules do not interfere with cell lysis after mitomycin C induction. Nonencapsulated variants of the 6 encapsulated *H.influenzae* type b strains that were not able to undergo lysis after mitomycin C treatment remained uninducible after the loss of the capsule indicating that these strains were probably nonlysogenic. These data show that lack of lytic activity was unlikely to be due to inhibition of prophage induction by the capsule.

3.3.5. Frequency of lysogeny in encapsulated and nontypable strains of *Haemophilus influenzae*

Since none of the previously described *H.influenzae* phages (HPIcl, S2 and N3) were able to produce plaques on encapsulated *H.influenzae* type b strains, it was of interest to investigate whether or not capsules have an inhibitory effect on the ability of the cells to become lysogenised under natural conditions. The proportion of lysogenic strains among encapsulated *H.influenzae* type b isolates was compared with that of nontypable strains.

It was found that 4 out of 24 encapsulated type b strains and 7 out of 34 nontypable isolates were able to undergo lysis after mitomycin C induction and their lysates showed the presence of phage particles (see next chapter). These data suggest that possession of a capsule does not reduce the frequency of lysogenisation in nature. In contrast to our data, in 1978 Stuy reported that only 1 out of 92 encapsulated strains of *H.influenzae* clinical isolates was lysogenic for a defective phage related to HP3 phage of *H.influenzae* while 17 out of 71 nontypable isolates were lysogenic for either HP1-or HP3-defective phage. The author concluded that the *H.influenzae* capsule greatly reduces the ability of a given cell to become lysogenised. The discrepancy between the results reported here and those reported previously by Stuy is probably due to the fact that no inducing agents were used in the latter study. In addition, Stuy confined his study to defective phages that were genetically related to HP1 and HP3.

3.3.6. Properties of lysogenic strains

Information on the sources and biological properties of the lysogenic isolates of *H.influenzae* is given in Table 3.2. The recovery of lysogenic strains among healthy as opposed to sick children was similar and not related to the site of isolation. The lysogenic strains belonged to three different biotypes (I, II and V). Only one of the 16 strains that was lysed by mitomycin C was resistant to ampicillin due to the production of beta-lactamase.

4. MORPHOLOGICAL CHARACTERISATION OF *HAEMOPHILUS INFLUENZAE* PHAGES

4.1. Introduction

Previous studies on phage morphology have shown that most phages consist of two major parts: a head and a tail. The head represents a tightly packed core of nucleic acid surrounded by a protein coat or capsid. The protein capsid of the head is made up of identical subunits, packed to form a prismatic structure, usually hexagonal in cross section. Tails of bacteriophages are either contractile or noncontractile, or either long or very short. According to a scheme first proposed by Bradley (1967) and revised by Ackermann and Eisenstark (1974) bacteriophages can be divided into three basic morphological types.The most complex of these (type A) has a head with a hexagonal outline and a tail with a contractile sheath attached to it. The second group (type B) also has six-sided head and a longer noncontractile tail. The third group (type C) has a short noncontractile tail and a six-sided head. Each type may further be divided into three sub-types based on the shape of the phage heads which are either isometric (sub-type 1) or prolate (sub-type 2 and 3). Some phages lack tails altogether. In the morphological groups D and E, for example, the capsid is a simple icosahedron. Morphological group D phages consist of capsids with larger capsomeres compared with type E which have smaller capsomeres. Morphological group F phages are quite unlike the others, the virion being in the form of a long flexible filament and there are no additional structures of any kind attached to it. The group can be divided into sub-types according to their tail length (sub-type F1 and F2).

Four *H.influenzae* bacteriophages, HPIcl, S2 (Boling *et al.*, 1973), N3 (Samuels and Clarke, 1969) and a defective bacteriophage released from *H.influenzae* Rd (Stachura *et al.*, 1969; Boling *et al.*, 1973) have been characterised morphologically. HPIcl, S2 and a defective phage from strain Rd belong to morphological group A1 while N3 belongs to group B1 (Ackermann and Eisenstark, 1974). Data presented in the previous chapter demonstrated that sixteen out of 54 *H.influenzae* clinical isolates were able to undergo lysis after mitomycin C treatment, suggesting that these strains were probably lysogenic. Attempts were made to detect the presence of phage particles in the lysates and to describe their morphology.

4.2. Materials and Methods

4.2.1. Phage preparation

Lysates obtained after induction with mitomycin C (see Materials and Methods, Chapter 2) were centrifuged at 8000 rev/minute for 15 minutes for the removal of unlysed cells and cell debris. The supernantants were sterilised by filtration through 0.45μM Millipore filters (Millipore Corporation).

The phages were concentrated by centrifugating 12ml of lysate in a Beckman L8-55 Ultracentrifuge at 40000 rev/minute (110 000g) for two hours. The supernantant was removed and the pellet was resuspended in 100μl of 5% BHI broth supplemented with 1mM $CaCl_2$.

4.2.2. Electron microscopy

4.2.2.1. Grids

Copper Gilder grids (G 200H) were used.

4.2.2.2. Support film

A solution of 0.30% w/v formvar in chloroform was prepared in a glass beaker. The formvar was allowed to adhere to a slide as a thin film by dipping a clean slide into the formvar solution. The film was separated from the slide by carefully and slowly lowering of the slide at an angle of about 30^{o} into glass distilled water. The grids were mounted onto the floating film and were picked with a filter paper. The paper was allowed to dry and the grids were carbon coated.

4.2.2.3. Sample application and staining

Microdroplets of phage suspensions were pipetted onto the grid and allowed to adsorb for 2 - 10 minutes. Preparations were rinsed by touching the sample meniscus to water. The excess water was withdrawn by filter paper. Samples were negatively stained by placing a drop of 2% uranyl acetate (Ridel de Hoen, West Germany) on the grid and immediately withdrawing the excess stain with filter paper. The preparation was allowed to dry.

4.2.2.4. EM examination

The phage preparations were examined for the presence of virions with a transmission electron microscope (JEM 100S, Joel Ltd, Japan) at a magnification of 40000 - 100000. Pictures of the virions were taken by exposing Ilford electron microscope film (Ilford Ltd, England) for 1.5 seconds using the electron microscope built-in camera.

4.2.2.5. Micrographs

The films were developed in Ilford PQ Universal developer for 4 minutes, and fixed in Hypam fixer. The negatives were printed on Ilford speed paper, grade 4, using Omega D2 enlarger (Simmons, USA). Pictures were developed in Infospeed Multigrade paper developer and fixed in Ilform Hypam paper, both from Ilford Ltd, England. They were washed in water and dried. All measurements of the virions were done on the prints.

4.3. Results and Discussion

The presence of viral particles was detected in 11 out of 16 lysates. The phages were named "J" for Johannesburg. Numbers were assigned to the independently isolated phages. (Table 4.1)

The particles of all phages, except one, consisted of a hexagonal head and a tail. According to the tail morphology the phages belonged to two groups: A1 and B1(Ackermann and Eisenstark, 1974). Seven phages belong to group A1, and four to B1. The virion of group A1 phages had a hexagonal head and a long tail with repeating crosswise stripes. The tail terminated in a well defined tail plate. It was not possible to demonstrate tail fibres by uranyl acetate staining (Figures 4.1, 4.2, 4.3, 4.4, 4.5, 4.6 and 4.7). The phages from group B1 had a head similar to those of group A phages and longer noncontractile tails. Unlike A1 phages, no base-plate was detected, and tail fibres were not demonstrated by negative staining (Figures 4.8, 4.9, 4.10 and 4.11). In some phage preparations, incomplete phage particles with empty heads (ghosts) were present.

Table 4.1 Lysogenic isolates of *H. influenzae* recovered from healthy children and paitents.

H.INFLUENZAE STRAIN	SOURCE	SPECIMEN	SEROTYPE	BIOTYPE	PHAGE DESIGNATION
45	Healthy	TS[a]	NT[b]	V	J1
34	Healthy	TS	NT	I	J2
14	Healthy	TS	NT	V	J3
18	Healthy	TS	NT	I	J4
168	Patient	CSF[c]	b	I	J5
53	Healthy	TS	NT	II	J6 and J 12
122165	Patient	CSF	NT	II	J7
59	Healthy	TS	NT	II	J8
15831	Patient	CSF	b	II	J9
122160	Patient	CSF	b	II	J10
35910	Patient	CSF	b	I	J11

[a]TS = throat swab

[b]NT = nontypable

[c]CSF = cerebrospinal fluid

Table 4.2 Dimensions of *H.Influenzae* phages .

		HEAD DIMENSIONS (nm)		TAIL DIMENSIONS (nm)	
PHAGE	GROUP	LENGTH	WIDTH	LENGTH	WIDTH
J1	A	63 ± 5	58 ± 4	148 ± 8	20
J2	A	60 ± 1	60 ± 2	160 ± 2	20
J3	A	60 ± 2	60 ± 2	155 ± 4	20
J4	A	60 ± 2	60 ± 2	158 ±6	20
J5	A	55 ± 2	50 ± 0.5	135 ± 5	20
J6	A	60	50	70	20
J7	A	60 ± 2	58 ± 6	63 ± 7	20
J8	B	58 ± 3	57 ± 0.8	211 ± 5	10
J9	B	62 ± 3	58 ± 7	223 ± 17	10
J10	B	62 ± 2	62 ± 2	210 ± 4	10
J11	B	60 ± 1	60 ± 2	195 ± 5	10
J12	F			210 ± 10	10

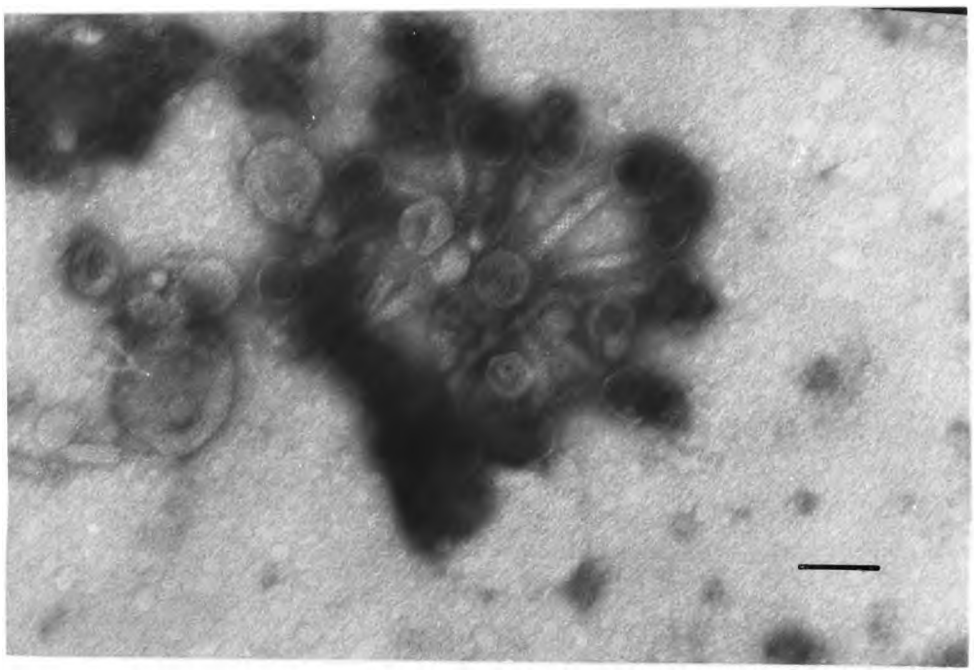


Figure 4.1 **Electronmicrograph of *H. influenzae* phage J1. Magnification x 100000; bar, 100nm.**

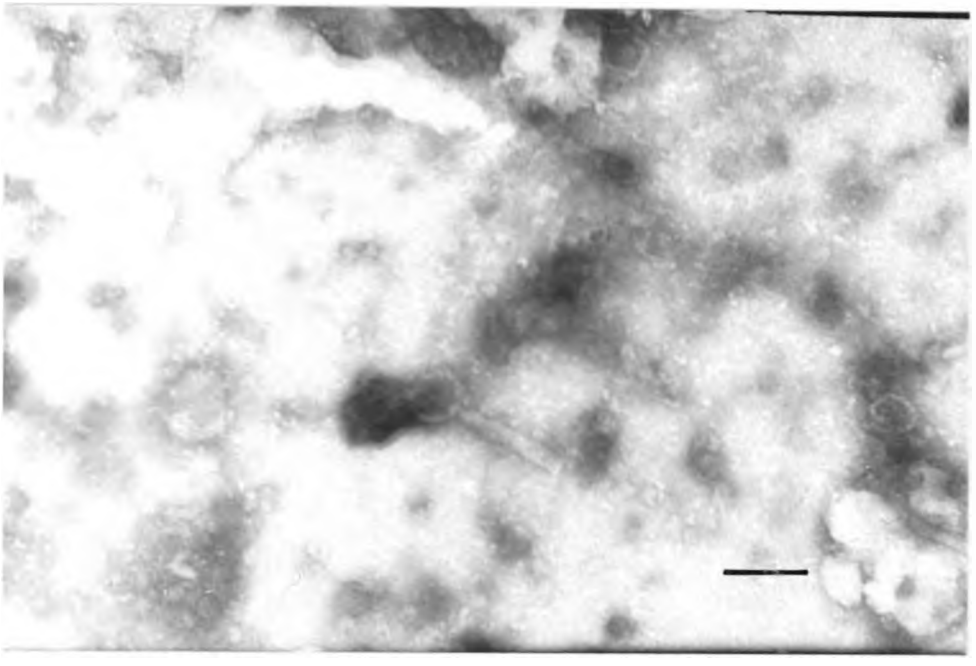


Figure 4.2 **Electronmicrograph of *H. influenzae* phage J2. Magnification x 100000; bar, 100nm.**

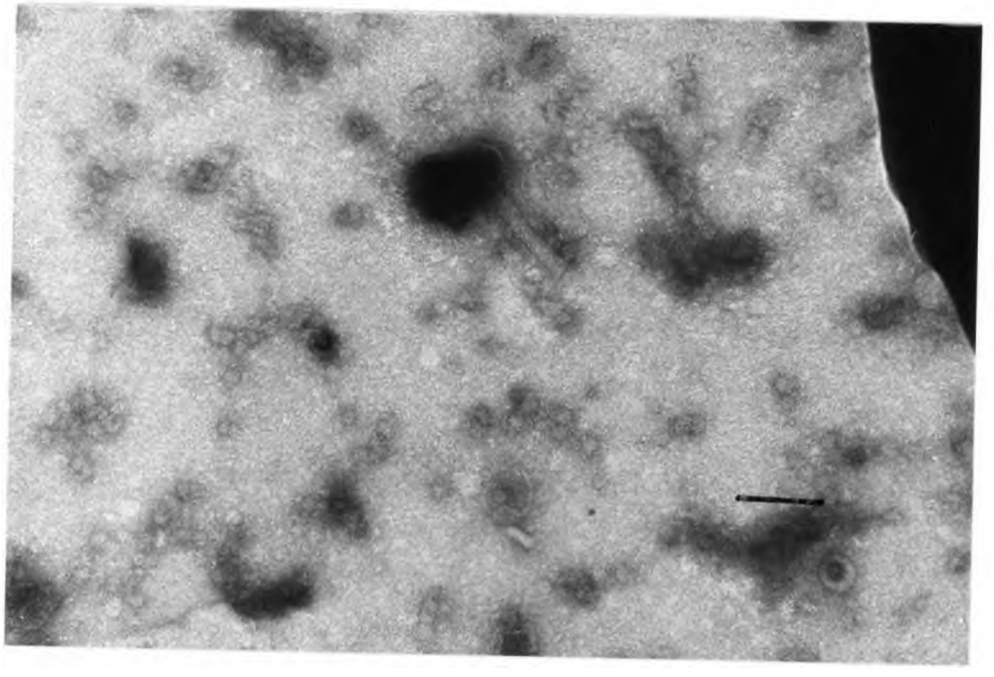


Figure 4.3 **Electronmicrograph of *H. influenzae* phage J3. Magnification x 100000; bar, 100nm.**

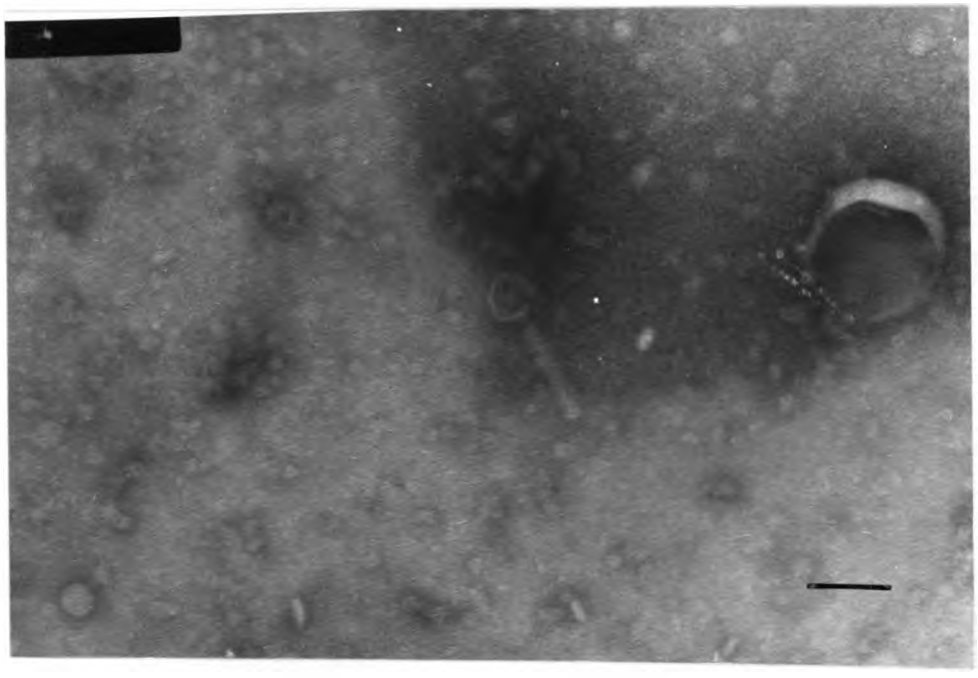


Figure 4.4 **Electronmicrograph of *H. influenzae* phage J4. Magnification x 100000; bar, 100nm.**

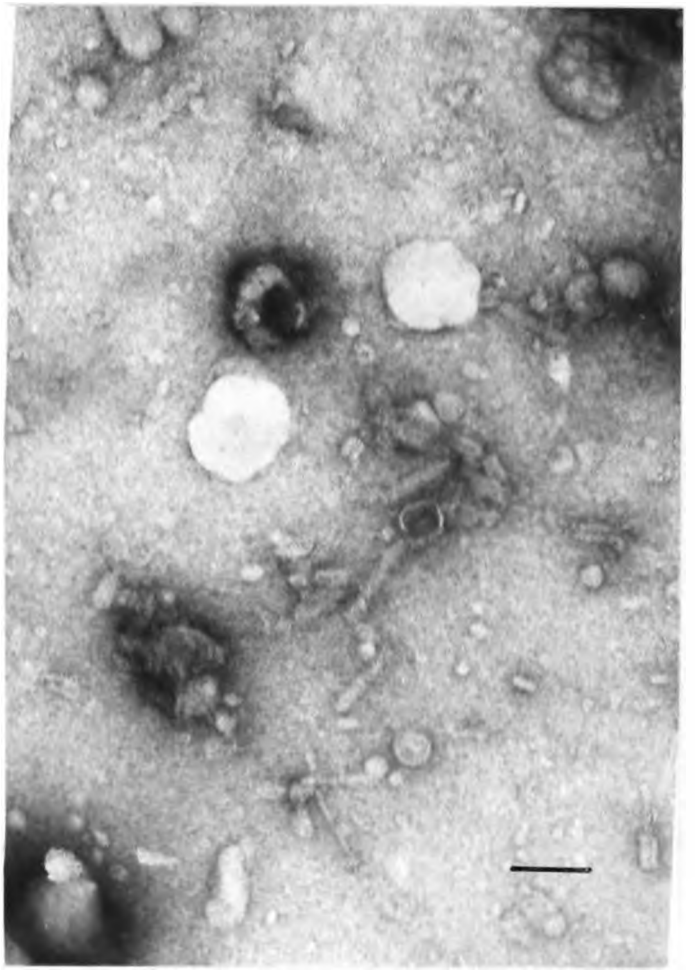


Figure 4.5 **Electronmicrograph of *H. influenzae* phage J5. Magnification x 100000; bar, 100nm.**

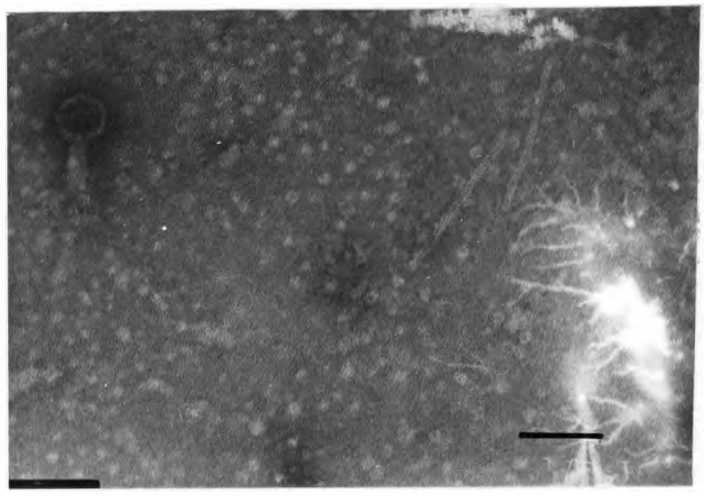


Figure 4.6 **Electronmicrograph of *H. influenzae* phage J6. Magnification x 100000; bar, 100nm.**

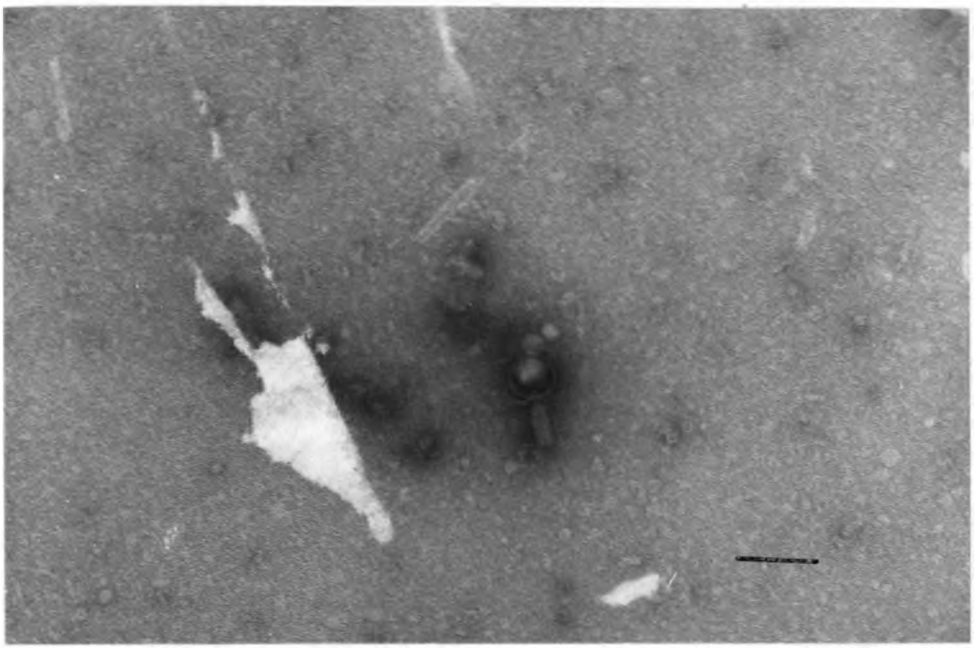


Figure 4.7 **Electronmicrograph of *H. influenzae* phage J7. Magnification x 100000; bar, 100nm.**

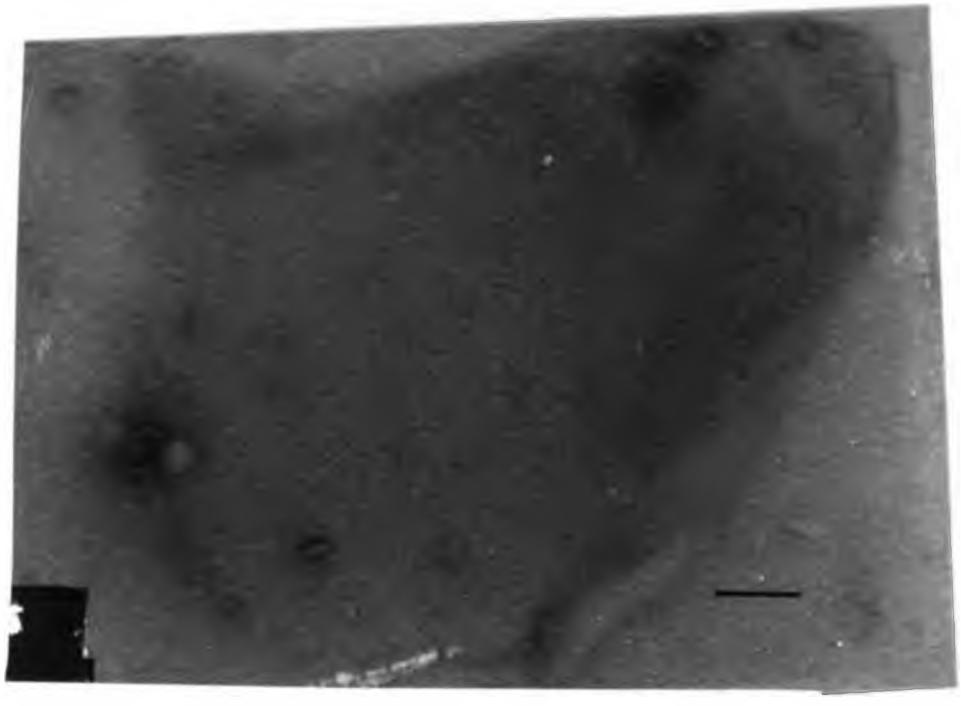


Figure 4.8 **Electronmicrograph of *H. influenzae* phage J8. Magnification x 100000; bar, 100nm.**

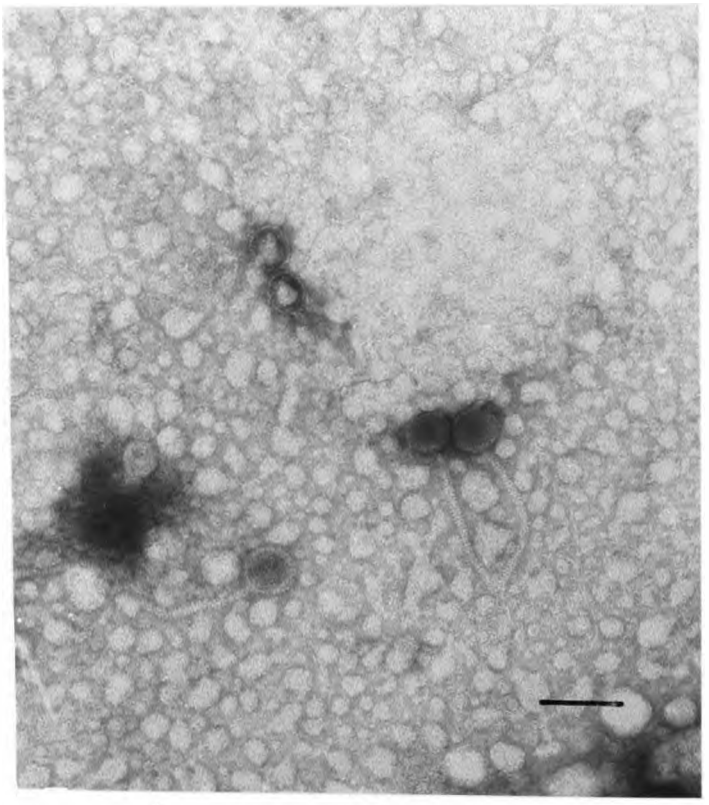


Figure 4.9 **Electronmicrograph of *H. influenzae* phage J9. Magnification x 100000; bar, 100nm.**

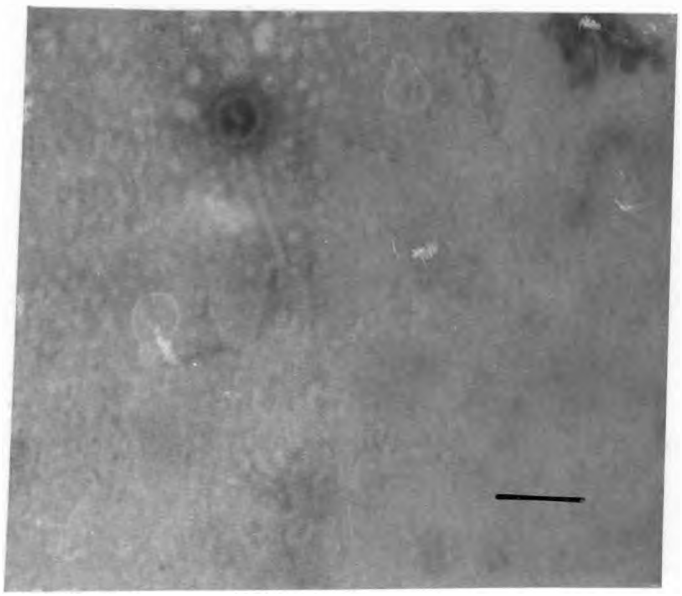


Figure 4.10 **Electronmicrograph of *H. influenzae* phage J10. Magnification x 100000, bar, 100nm.**

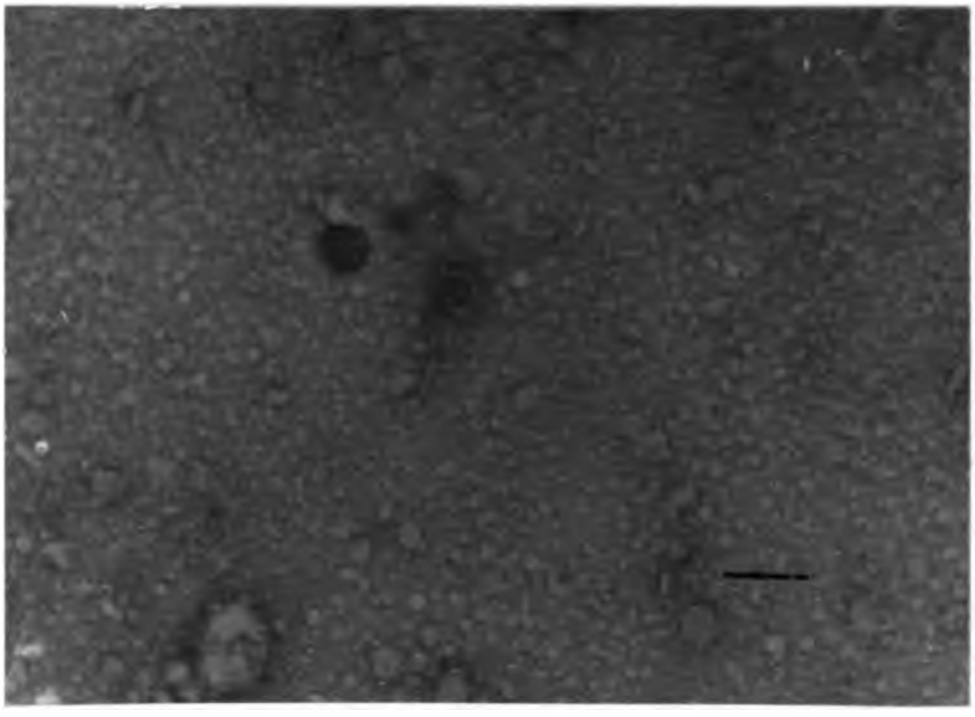


Figure 4.11 **Electronmicrograph of *H. influenzae* phage J11. Magnification x 100000; bar, 100nm.**

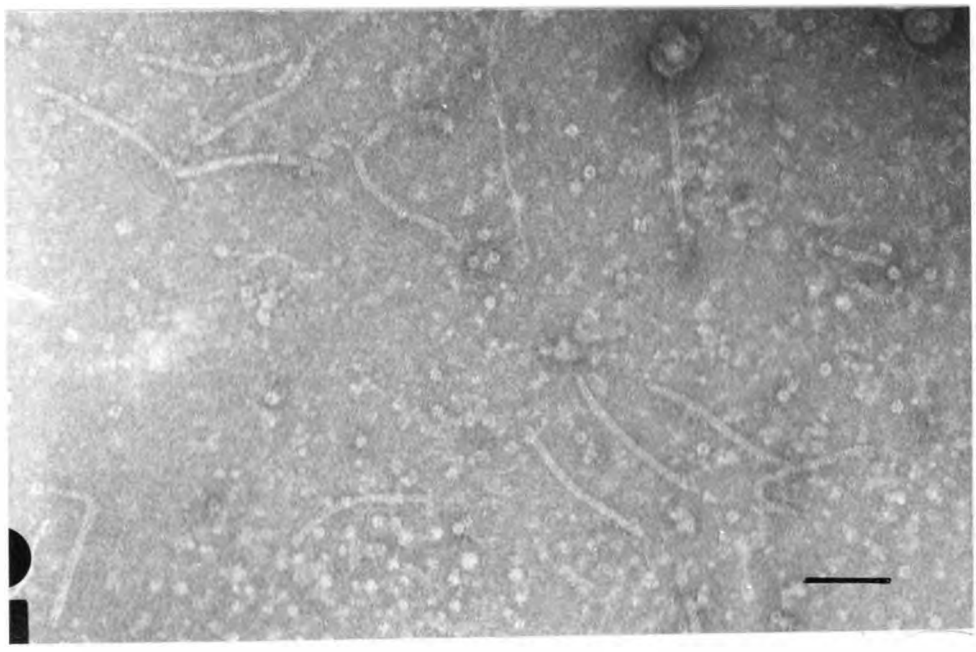


Figure 4.12 **Electronmicrograph of *H. influenzae* phage J12. Magnification x 100000; bar, 100nm.**

The dimensions of the components of the phages recovered from *H.influenzae* lysogenic strains are presented in Table 4.2. Phages from the same morphological group showed only slight differences in dimensions of their heads and tails. The morphological features of the phages from group A1 described here were similar to those of *H.influenzae* phages HP1, S2, and the defective phage from strain Rd that also belong to this group (Boling *et al.*, 1973). Only one type B phage was previously described in *H.influenzae* and this phage shows a similar morphology to that of the four type B phages described here (Samuels and Clarke, 1969). The lysate of *H.influenzae* strain 53 revealed the presence of two types of phage particles designated J6 and J12 (see Table 4.1 and Fig. 4.6 and 4.12). While J6 belonged to group A1, phage J12 consisted of rod-shaped particles that had not been previously described in *H.influenzae*. The latter resembled tails of group B phages. It is also possible that these particles represent filamentous phages (group F).

The data in Tables 4.1 and 4.2 show that the presence or absence of a capsule appears to correlate with the morphological type of the phage present. Thus, group B1 phages were most common among encapsulated *H.influenzae* type b strains, while the most common morphological type among nontypable *H.influenzae* strains was type A1. However, the two morphological types may occur in both encapsulated and nontypable *H.influenzae*. A larger number of strains have to be examined in order to assess the relationship between capsular type of the host and the morphological type of the corresponding phage.

The inability to detect phages in 5 lysates after electron microscopic examination was probably due to the release of a small number of phages. An alternative explanation for the lack of phage particles is that in these strains lysis was not related to the presence of inducible prophages but to another unknown mechanism. Although the inhibitory effect of chloramphenicol on cell lysis in these strains favours the prophage hypothesis, the possibility cannot be excluded that chloramphenicol may exhibit a similar effect on other lysis-inducible factors.

5. BIOLOGICAL PROPERTIES OF *HAEMOPHILUS INFLUENZAE* BACTERIOPHAGES

5.1. Introduction

In his early experiments on the bateriophages, d'Herelle had observed "taches vierges", plaques or negative colonies in areas of dense bacterial growth on the nutrient agar surfaces. D'Herelle found that these plaques represent regions in which bacteria had been destroyed by the multiplication of the bacteriophage present in the bacterial inoculum. The plaque size and morphology are characteristic features for a particular phage-bacterium combination, and may vary for different phages grown on different bacterial hosts. Virulent phages form clear plaques, whereas temperate phages form turbid plaques, because of the overgrowth of lysogenised bacteria in the plaque.

The host range of the phage is often restricted to strains from a single bacterial species, but exceptions to this rule are common. A *Yersinia pestis* phage, for instance, also lyse some *Salmonella* and *Shigella* species (Lazarus and Gunnison, 1947). The specificity of the phage-bacterium relationship is in most cases determined by the adsorption process. Susceptibility or resistance to certain groups of phages is dependent on the presence or absence of receptors on the surface of bacterium for those phages. Strains suceptible to a given phage are called indicator strains. Changes in the surface of a bacterium, for example by encapsulation or through the loss of some surface antigens may block the adsorption of a phage otherwise able to multiply in such a host. Multiplication of phage in a given host may be blocked at a stage that follows adsorption. This is often a consequence of the lysogenic condition of the host. The phage repressor usually inhibits the vegetative replication of homologous or genetically related phages. In addition, the presence of some restriction systems in the host may prevent the production of plaques due to degradation of phage DNA.

The hosts for *H.influenzae* bacteriophages HPIcl and S2 are all rough segregants of typable strains from different serotypes of *H.influenzae* (Piekarowicz and Glover, 1972; Stuy, 1978) while the only host for *H.influenzae* N3 phage is strain Rd9 (Samuels and Clarke, 1969).

In this study the ability of encapsulated and nontypable clinical isolates of *H.influenzae* to undergo lysis after mitomycin C induction was demonstrated

(Chapter 2) and the presence of phage particles in these lysates was confirmed electron-microscopically. Attempts were made to find a suitable indicator for the inducible phages recovered from *H.influenzae* clinical isolates and to study some of the biological properties of the phages.

5.2. Materials and Methods

Sixteen lysates prepared by mitomycin C induction were used for testing the ability to form plaques.

5.2.1. Spot test for phage sensitivity

Frozen cultures of *H.influenzae* were diluted 1 to 40 in supplemented BHI broth and grown at 37°C for 18 hours. Aliquots of 0.2ml of each culture were added to 2.5ml soft agar (0.7%). The mixture was poured onto the surface of BHI agar (1.4%) supplemented with the growth factors plus 1mM of both $MgCl_2$ and $CaCl_2$. Lysates were then spotted on the surface of the soft agar in 10μl aliquots and plates incubated overnight at 37°C. Spot tests with *Streptococcus pneumoniae* were performed on Todd Hewitt agar (Oxoid Limited) supplemented with 10% yeast extract.

5.2.2. Assay for killing ability of phage lysates

Overnight *H.influenzae* cultures were diluted 1 to 40 in BHI broth and grown at 37°C to an OD of 0.1 at 650nm. The cultures were diluted in BHI broth to a cell density of 10^3 CFU/ml. 0.1ml of fresh lysate was added to 0.9ml diluted culture, and the mixture was incubated at 37°C. Samples were taken at 5 minutes intervals for determination of CFU's.

5.2.3. Test for transmissibility of factors inhibiting growth of indicator strains

BHI agar from clear spots produced by the lysates was removed with a Pasteur pipette and resuspended in 1ml broth. The suspension was spotted again on the same indicator as described above.

5.2.4. Heat inactivation of the factors inhibiting baterial growth

One milliliter of the lysates was heated at 40°C, 50°C, 55°C and 60°C for 30 minutes and the lysates were plated on the strain that was inhibited.

5.2.5. Protease sensitivity of factors inhibiting bacterial growth

To 0.1ml aliquots of each lysate, 1μl, 2μl, 5μl, 10μl and 20μl of a stock solution of 1mg/ml of protease was added. After incubation for 1 hour at 37°C the lysates were spotted on the strain which showed clearing.

5.2.6. Examination of the lysates for ability to lysogenise the indicator strain

Bacteria from the centre of a zone with inhibited growth were resuspended in BHI broth and plated on BHI agar for single colonies. Several colonies (Table 5.3) were resuspended into supplemented BHI broth and grown to OD of 0.1 at 650nm. Mitomycin C was added to a final concentration of 0.5μg/ml and the culture incubated at 37°C. The OD was determined at 60 minutes intervals.

5.2.7. Curing procedures

Mitomycin C treatment : An overnight BHI broth culture of a lysogenic *H.influenzae* strain was diluted 1:40 in BHI broth and grown at 37°C with shaking to OD of 0.1 at 650nm. Mitomycin C (3μg/ml) was added to the culture followed by 30 minutes incubation at 37°C. The cells were centrifuged and resuspended in supplemented BHI broth. After overnight incubation at 37°C the culture was plated in BHI agar for single colonies and incubated for 18 hours at 37°C. Two hundred colonies were tested for their ability to lyse after mitomycin C treatment and 700 colonies were examined for sensitivity to the homologous phage by the spot test.

UV Light treatment : Exponentially growing lysogenic cells in BHI broth were centrifuged and resuspended to the initial volume in saline. Five ml portions were irradiated with UV light (Toshiba 15-W germicidal lamp) for 10 minutes. The cell survival was 0.01%. The culture was plated for single colonies. The same numbers of colonies as described above were tested for lysis after mitomycin C reatment and for sensitivity to the homologous phage.

Heat treatment : The lysogenic culture to be cured from the prophage was grown by shaking in BHI broth at 34°C to an OD of 0.1 at 650nm. The culture was then diluted in BHI broth and incubated for 5 minutes at 42°C followed by transfer to 34°C for 2 hours. The culture was plated for single colonies. After 24 hours incubation at 34°C, 281 colonies were tested for ability to lyse after treatment with mitomycin C by following OD at 1 hour intervals.

5.2.8. Assay for direct adsorption

Concentrated phage lysates were added to homologous lysogenic strains grown to an OD of 0.6 in BHI broth with 1mM $CaCl_2$, 1mM $MgCl_2$ and trytophan(0.1 %.)The mixture was incubated for 10 minutes at 37°C, and a droplet of the mixture was negatively stained on the grid. The latter was examined under EM for the presence of phage particles that are attached to the cell.

5.2.9. Assay for indirect adsorption

Encapsulated and nonencapsulated variants of *H.influenzae* were grown in BHI broth at 37°C to an OD of 0.6 at 650nm. The cells were sedimented by centrifuging 15ml of culture at 10000 rev/minute for 10 minutes. The supernatant was discarded and 2.5ml of fresh lysate obtained after mitomycin C induction was added to the cells. The mixture was incubated for 10 minutes at 37°C and the cells sedimented by centrifugation of 5000 rev/min for 10 minutes. A grid was prepared from the supernatant, negatively stained with 2% uranyl acetate and viewed under EM to assess the amount of unadsorbed phages. A grid of lysate which was not mixed with cells was included as a control in order to determine the initial concentration of the phage.

5.3. Results and Discussion

5.3.1. Attempts to find suitable hosts for *Haemophilus influenzae* phages

All lysates obtained after mitomycin C induction were tested for their ability to form plaques initially on 12 *Haemophilus* strains including encapsulated type b isolates (smooth), nonencapsulated (rough) variants derived from the encapsulated strains Rb, Rc and Rd and nontypable strains of *H.influenzae* (Table 5.1). In addition, *Haemophilus aegyptius* and *Haemophilus parainfluenzae* strains were also examined for phage sensitivity. *H.influenzae* type e and f were not included because they possess strong restriction systems (Piekarowicz and Kalinoska, 1974).

None of the phages was capable of producing plaques on the above-mentioned strains and an additional 102 strains subsequently tested were not phage sensitive (Table 5.2). Several heterologous Gram-negative and Gram-positive species that may be present in the upper respiratory tract

Table 5.1 ***H. influenzae*** **strains tested initially for phage sensitivity.**

STRAIN	SEROTYPE	SOURCE
H.influenzae 56	b	CSF[c], patient
H.influenzae 11604	b	CSF, patient
H.influenzae 5R	NEV[a]	CSF, patient
H.influenzae 14681R	NEV	CSF, patient
H.influenzae 12	NT[b]	TS[c] Healthy Child from Creche
H.influenzae 02523	NT	Blood, patient
H.influenzae Ra	NEV	G. Leidy
H.influenzae Rc	NEV	G. Leidy
H.influenzae Rd	NEV	J. Setlow
H.influenzae 436	b	
Haemophilus aegyptius		NCTC[d]
Haemophilus parainfluenzae Fidles		NCTC

[a]Nonencapsulated varient

[b]Nontypable

[c]Cerebrospinal fluid

[d]National Collection of Type Cultures, London

Table 5.2 Strains tested for plaque-forming ability of *H. influenzae* phages.

STRAIN	NO. OF STRAINS TESTED	SOURCE
H.influenzae Ra	1	G. Leidy
H.influenzae type b	24	SAIMR[a]
H.influenzae Rc	1	G. Leidy
H.influenzae Rd	1	J. Setlow
H.influenzae non-typable	51	SAIMR
H.aegyptius 8502	1	NCTC[b]
H.aphrophilus R5906	1	NCTC
H.parainfluenzae	10	SAIMR
Escherichia coli	2	SAIMR
Klebsiella pneumoniae	6	SAIMR
Proteus mirabilis	1	ATCC[c]
Proteus vulgaris	1	SAIMR
Streptococcus pneumoniae R6	1	S. Lacks
Enterococcus faecalis	1	SAIMR
Streptococcus salivarius	2	SAIMR
Staphylococcus aureus	10	SAIMR

[a]South African Institute for Medical Research

[b]National Collection of Type Cultures, London

[c]American Type Culture Collection

were also included for testing for lysis by the inducible phages recovered from *H.influenzae* and they were also negative.

5.3.2. Killing effect of *Haemophilus influenzae* phages

Four phage lysates (J1, J2, J3 and J6) obtained after mitomycin C induction of nontypable lysogenic strains and one lysate (J7) from an encapsulated type b strain were able to inhibit the growth of nontypable strains of *H.influenzae*. This was presented by the occurrence of clear spot on the indicator strain. The host range of these lysates is given in Table 5.3. The strain harbouring inhibitory factor was resistant to the homologous factor.

In order to study whether the inhibition was due to an effect on cell growth or to killing of the bacterial cells, the number of bacteria was monitored after addition of phage lysate and incubation at 37°C. The curve of the surviving *H.influenzae* 12B bacteria at different times is presented in Fig 5.1. After 20 minutes incubation with J1 lysate about 95% of the cells were killed. The factor in the lysate that was responsible for the killing effect was confined to the sediment containing the bacteriophage after centrifugation and was therefore likely to be phage-associated. Unlike the lytic bacteriophages this factor was nontransmissible (see Materials and Methods section.). The inhibitory effect present in the other phage lysates also sedimented with the bacteriophage and was due to cell killing. The killing factors were resistant to chloroform, but were inactivated by proteolytic enzymes and after heating at 60°C for 30 minutes. A similar killing effect was observed by Stuy (1978) when phage HP1 was spotted on nontypable *H.influenzae* isolates. Examination for the ability of phage lysates exhibiting a killing effect to lysogenise sensitive cells showed that they lack such ability. None of the surviving colonies picked from the phage spot was able to undergo lysis after treatment with mitomycin C (Table 5.4).

The probable reason for the inability of the group of 5 inducible phages exhibiting a killing effect to lysogenise cells is that the phages kill the sensitive bacteria upon contact before the DNA becomes expressed.

Table 5.3 Killing effect of *H.influenzae* phages .

PHAGE	*H. INFLUENZAE* STRAINS INHIBITED BY THE PHAGES
J1	12B, 19W,7W,4W,20W,B241
J2	12B, 19W, 7W, 4W, 20W, B241
J3	48B, 32B, 33B, 37B, 13B
J6/j12	B241
J7	4W, 7W, 19W

Table 5.4 Test for lysogeny.

PHAGE	INDICATOR USED	NUMBER OF COLONIES TESTED
J1	12B	328
J2	12B	245
J3	32B	156
J6/J12	45I	206
J7	4W	190

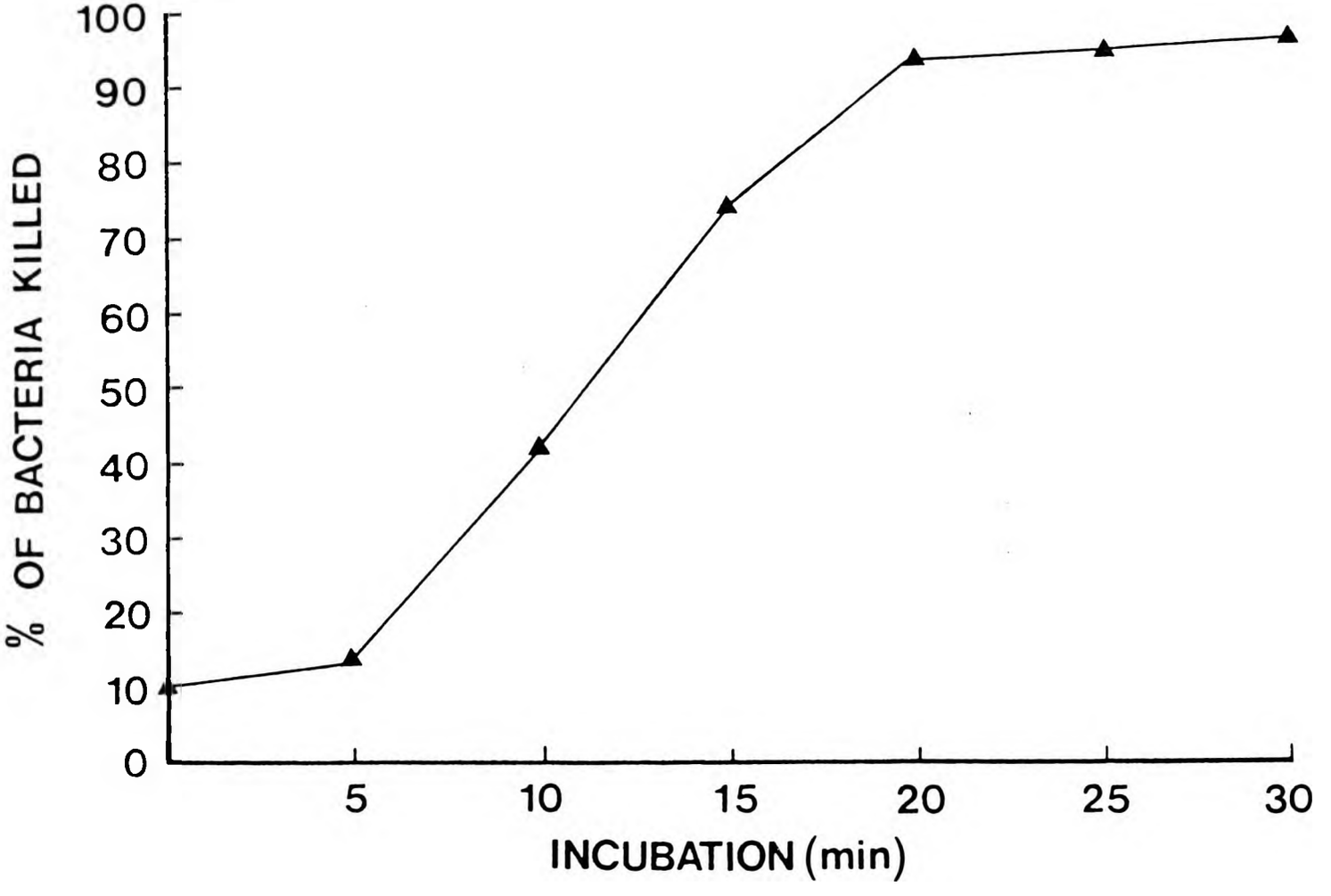


Figure 5.1 Kinetics of killing of *H. influenzae* by lysate from *H. influenzae* 45.

5.3.3. Attempts to cure *Haemophilus influenzae* strains from their prophage

None of the colonies of *H.influenzae* 15831 and 45B that survived heavy exposure to UV light and the colonies examined after mitomycin C treatment was cured of the prophage. Heat shock curing previously demonstrated to be effective in curing *E.coli* lysogenic strain HfrU 1858thy- (Weisberg *et al.*, 1967) from its prophage also failed to cure *H.influenzae* strains from the prophage, probably because their repressors are not sensitive to heat. Failure to cure the lysogenic strains from the prophage suggests that the lysogenic state, at least in these strains, was very stable.

5.3.4. Study of phage adsorption

Because of the inability of *H.influenzae* inducible phages to produce plaques on a large number of *H.influenzae* strains, it was of interest to examine the ability of the phages to attach to the receptors of homologous cells. Electron microscopic observation of 2 phages (J1 and J9) to adsorb to homologous lysogenic cells showed that they failed to attach to the cells. Figures 5.2 and 5.3 show encapsulated *H.influenzae* 15831 as well as its nonencapsulated variant exposed to the homologous phage J9. Electron microscopic observation showed that most phages were not attached to the cells. Only a few phages appeared to be attached to the cells which could have been a fortuitous observation as a result of phages floating around the cells. Furthermore there was no decrease in phage concentration in the supernatant obtained after centrifugation of a mixture of phage and bacteria following a brief period of incubation. The lack of adsorption of these phages cannot be explained. Normally, lysogenic cells are able to adsorb the phage they carry in the lysogenic state, but exceptions to this rule cannot be ruled out. A possible explanation for the lack of adsorption could be that the tails of these phages are defective.



Figure 5.2 Electronmicrograph of encapsulated *H. influenzae* 15831 exposed to the homologous phage J9.

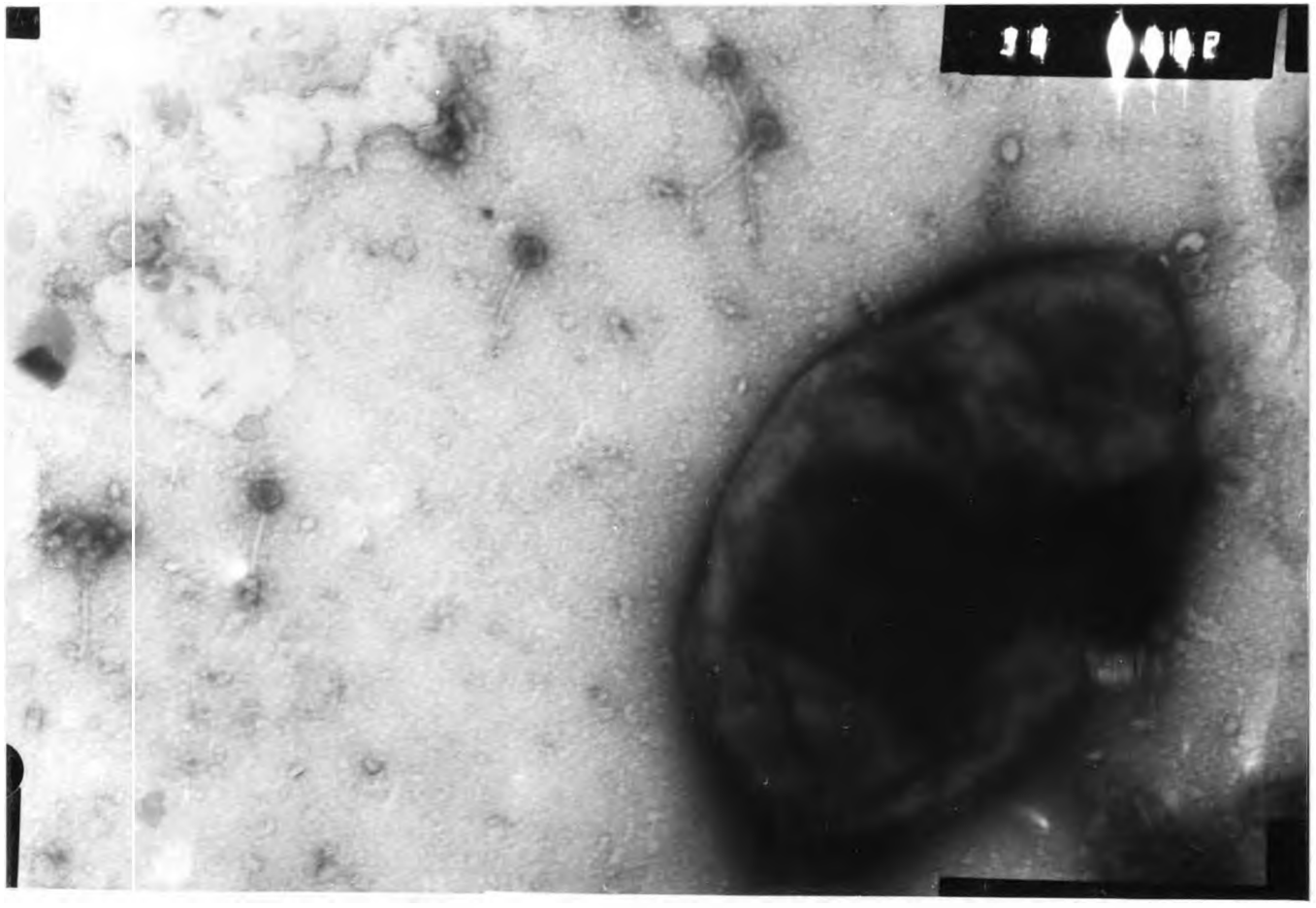


Figure 5.3a Electronmicrograph of nonencapsulated *H. influenzae* 15831 exposed to the homologous phage J9.

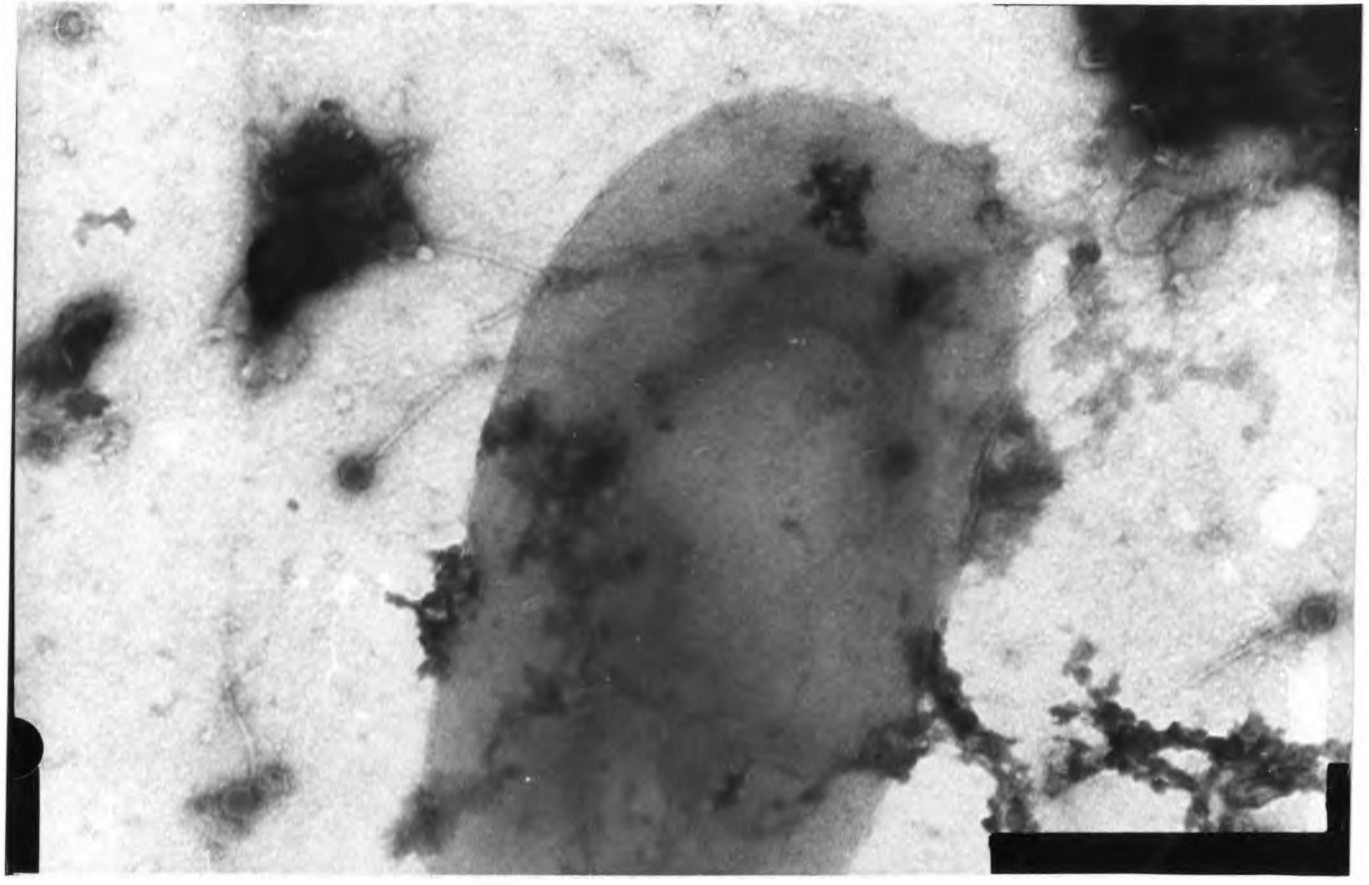


Figure 5.3b Electronmicrograph of nonencapsulated *H. influenzae* 15831 exposed to the homologous phage J9 .

6. TRANSFORMABILITY OF LYSOGENIC STRAINS OF *HAEMOPHILUS INFLUENZAE*

6.1. Introduction

The presence of prophage can inhibit transformation in several bacterial systems. Possible explanations for the inhibition of transformation in lysogenic cultures include:

- restriction of unmodified DNA due to the presence of genes on the prophage conferring synthesis of restriction enzyme;
- deficiencies in recombination enzymes or enzymes needed in transformation induced by the prophage;
- alterations of cell wall or membranes resulting in reduced uptake of bacterial DNA;
- preferential lysis of competent cells by their prophages, and
- degradation of bacterial DNA by unspecific nucleases encoded by prophage genes.

In lysogenic streptococci (Parsons *et al.*, 1973) this inhibition is due to inability of the cells to develop competence as judged by the lack of irreversible binding of transforming DNA. *Bacillus subtilis* lysogenic cells also show reduced level of transformation, however the ability of cells to become competent is retained (Yasbin *et al.*, 1973; Yasbin *et al.*, 1975). In the latter case transfection by phage DNA is hardly affected and may even be increased. The reduction in the frequency of transformation in *B.subtilis* is attributed to the selective elimination of transformed lysogenic cells from the population of competent cells due to the release of bacteriophage.Transformation of *Haemophilus influenzae* is not appreciably altered if prophage HPlcl or S2 is present in the recipient (Sedgwick *et al.*, 1975; Setlow *et al.*, 1973). Similarly the presence of defective prophage in *H.influenzae* Rd does not influence the transforming ability of this strain (Setlow *et al.*, (1973). *H.influenzae* Rd9 lysogenic for temperate bacteriophage N3 was found to be virtually nontransformable and nontranfectable (Piekarowicz and Siwinska, 1977). This inhibition was partly due to the decreased ability of the cells to develop competence, and partly due to some events taking place after adsorption of DNA.

The lack of competence is explained by the reduced ability of the cells to bind DNA irreversibly.

Since most of the encapsulated *H.influenzae* type b strains tested for transformability by Rowji *et al.*, (1989) were nontransformable, an attempt was made to investigate whether the presence of prophage had an inhibitory effect on DNA transformation in *H.influenzae* lysogenic strains.

6.2. Materials and Methods

6.2.1. Bacterial strains

A total of 16 *H.influenzae* strains (10 encapsulated and 6 nontypable), that were able to lyse after treatment with mitomycin C were examined. Twelve nonlysogenic strains taken at random were used as controls.

6.2.2. Preparation of donor DNA

Purified DNA was prepared by the chloroform/octonal method (Goodgal, 1968).

6.2.3. Preparation of competent cells

Competence was induced by the static aerobic procedure (Gromkova *et al.*, 1989). This technique is similar to that described for the induction of competence in *Haemophilus parainfluenzae* (Gromkova and Goodgal, 1979). Overnight cultures grown in BHI broth were diluted 1:50 into supplemented BHI broth. Samples (10ml) were incubated in 90mm plastic Petri dishes at 37°C without shaking to the early stationary phase (OD of 0.6 at 650nm).

6.2.4. Transformation procedure

DNA at saturating concentration (0.3μg/ml) was added to 1ml BHI broth supplemented with 1mM $MgSO_4$. After addition of 0.1ml competent cells, the mixture was incubated for 40 minutes at 35°C without shaking and different dilutions were plated in triplicate for transformants, using an overlay method.

6.3. Results and Discussion

In order to study the effect of lysogeny on transformation, the frequencies of transformation of lysogenic *H.influenzae* isolates were compared with those of the non-lysogenic strains. Table 6.1 shows the transformation frequencies of the streptomycin resistance (str^r) marker in lysogenic *H.influenzae* recipient cells. Nine out of 16 lysogenic strains were nontransformable (transformation frequencies of less than 10^{-8}). Two strains exhibited reduced transformation frequencies (10^{-6} to 10^{-7}). Only 3 nonlysogenic *H.influenzae* strains were nontransformable while 9 were transformable (Table 6.2). The finding that most lysogenic *H.influenzae* isolates were nontransformable suggests that lysogeny may have inhibitory effect on transformation in these strains. However, the transforming ability of isogenic nonlysogenic and lysogenic strains was not compared because of the inability to cure the lysogenic strains from the prophage or to lysogenise *H.influenzae* in vitro. Unlike *B.subtilis* a decrease in the survival of lysogenic cells after development of competence or after addition of transforming DNA was not observed in this study. These findings show that at the physiological state of competence, lysogenic *H.influenzae* strains are not more susceptible to lysis. Thus, it seems, lysis of competent or transformed cells can be excluded as the cause for the inhibition. No attempts were made to determine whether the inhibition of transformation was related to a block in the irreversible binding of DNA. Another possible reason for the lack of transformation could be the loss or the impairment of one of the enzymes necessary for recombination as a result of prophage integration. Transformation in *H.influenzae* is known to require the presence of a host recombination system (Notani and Setlow, 1974). It is of importance to note that the lysogenic strains were inducible by mitomycin C and UV light which also requires a host recombination system.

It appears that inhibition of transformation by temperate phage is not a general phenomenon among *H.influenzae* clinical isolates. Five *H.influenzae* isolates which carry temperate phages were able to undergo efficient transformation. These findings suggest that inhibition of transformation ability by lysogeny depends on the type of prophage carried by the recipient. All but one lysogenic *H.influenzae* clinical isolate carrying morphological group B1 prophages (similar to N3 bacteriophage of *H.influenzae*) were nontransformable, irrespective of the presence or absence of capsule (Table 6.1). Only one *H.influenzae* clinical isolate lysogenic for the

morphological group A1 prophage (similar to HPIcl, S2 and a defective phage from *H.influenzae* Rd) was nontransformable.

It seems unlikely that the lack of competence in *H.influenzae* isolates is due solely to the presence of prophages. This conclusion was supported by the finding that non-transformable *H.influenzae* strains exist that are not inducible by mitomycin C (Table 6.1) and do not appear to carry prophages.

Table 6.1 Transformation of lysogenic *H.influenzae* strains.

RECEPIENT STRAIN	SEROTYPE	HOMOLOGOUS PHAGE	MORPHOLOGICAL GROUP	FREQUENCY OF TRANSFORMATION
45B	NT[a]	J1	A	$2x10^{-7}$
34B	NT	J	A	$4x10^{-4}$
14W	NT	J3	A	$1X10^{-4}$
18W	NT	J4	A	$9X10^{-6}$
168	b	J5	A	$1X10^{-5}$
53	NT	J6/J12	A	$3X10^{-3}$
122165	NT	J7	A	$< 10^{-8}$
59	b	J8	B	$< 10^{-8}$
15831	b	J9	B	$< 10^{-8}$
122160	b	J10	B	$< 10^{-8}$
35910	b	J11	B	$6x10^{-4}$
14981	b	PNI[b]		$< 10^{-8}$
14945	b	PNI		$< 10^{-8}$
1	b	PNI		$< 10^{-8}$
11	b	PNI		$< 10^{-8}$
13	b	PNI		$< 10^{-8}$

[a]Nontypable

[b]Phage not seen under EM

Table 6.2 Transformation frequencies of nonlysogenic *H.influenzae* isolates.

RECEPIENT STRAIN	SEROTYPE	TRANSFORMATION
2457	b	$< 10^{-8}$
77077	b	$< 10^{-8}$
1220155	b	$5X10^{-3}$
126814	b	$2X10^{-3}$
12	b	$3X10^{-2}$
10674	b	$6X10^{-2}$
64	b	$1X10^{-4}$
14981	b	$< 10^{-8}$
32B	NT[a]	$6X10^{-3}$
37B	NT	$2X10^{-2}$
12B	NT	$6X10^{-4}$
145	b	$2x10^{-2}$

[a]NT nontypable

7. CONCLUSIONS

One of the objectives of this study was to investigate whether the ability of *H.influenzae* clinical isolates to undergo autolysis in broth cultures as reported in the literature (Riley, 1981) was due to the presence of prophages. The results recorded in this dissertation showed that overnight BHI broth cultures of *H.influenzae* strains did not reduce greatly their optical densities. However, both lysogenic and nonlysogenic isolates exhibited significant loss in viability (10^2 to 10^3 times). This suggests that spontaneous production of phages from lysogenic strains was not responsible for the pronounced loss in viability of *H.influenzae*. Our data do not support the contension that *H.influenzae* undergo significant autolysis in BHI broth.

We were able to induce lysis in 16 out of 58 *H.influenzae* isolates after treatment with mitomycin C or after UV irradiation, while heat treatment was not efficient in lysing the cultures. Addition of chloramphenicol inhibited lysis by mitomycin C in all lysogenic strains tested by us, indicating that synthesis of new proteins was essential for the induction process. Studies on the effect of serotype b capsule on prophage induction and cell lysis have provided evidence that the capsule of *H.influenzae* do not interfere either with prophage induction or cell lysis after treatment with mitomycin C. According to our knowledge there are no previous reports on the effect of chloramphenicol and serotype b capsule on prophage induction and cell lysis in *H.influenzae*. In addition, we established that the recovery of lysogenic strains among encapsulated compared with nontypable isolates was similar, suggesting that possession of a capsule does not reduce the frequency of lysogenisation in nature. This result was unexpected because none of the previously described *H.influenzae* temperate phages was able to produce plaques on encapsulated *H.influenzae* type b strains. In 1978 Stuy found that only one out of 92 encapsulated strains of *H.influenzae* was lysogenic and concluded that a capsule greatly reduces the ability of a given cell to become lysogenised. However, since no inducing agents were used in Stuy's study, it is possible that the frequency of lysogeny among encapsulated *H.influenzae* was not estimated properly. In order to explain how lysogenisation occurs with phages that are not active against encapsulated *H.influenzae* bacteria, one can assume that the cells have been lysogenised during a period when capsule has not been phenotypically expressed or that lysogenisation occur by transfection with phage DNA. The latter does not require the presence of phage receptors on the cell surface. Although *H.influenzae*

is known to possess a highly efficient transformation system, attempts to lysogenise in vitro *H.influenzae* competent cells using DNA from phages S2, HP1 and N3 have been unsuccessful (Boling *et al.*, 1972; Jablon'ska and Piekarowicz, 1976). However, one can not rule out the possibility that such lysogenisation may occur in nature. An alternative hypothesis for the origin of the lysogenic encapsulated bacteria is that the capsular genes are carried by the phage.

Presence of phage particles was confirmed by electron microscopy in 11 out of 16 lysates. One lysate contained two different types of viral particles. This is the first large group of *H.influenzae* inducible phages that has been characterised morphologically. Only four *H.influenzae* phages were studied previously (Boling et *al.*, 1973; Samuels and Clarke, 1969). The virions of most phages recovered by us in *H.influenzae* consist of a hexagonal head and a tail. According to the morphology of their tails the phages belong to two groups as classified by Ackermann and Eisenstark (1974). One phage has rod-shaped particles and belongs to the morphogical group F. *H.influenzae* phage from group F has not been described previously.

None of the phages described here was able to form plaques when tested on large number of *H.influenzae* strains. However, the lysates of 5 group A1 phages were able to inhibit the growth of several nontypable *H.influenzae* strains. We were able to demonstrate that this effect was due to the killing of the sensitive cells. The factor responsible for the killing was resistant to chloroform but was inactivated by protolytic enzymes and after heating at 60°C for 30 minutes. The killing effect sediments with the bacteriophage and appears to be phage-associated. The inability of the group of 12 inducible phages recovered from *H.influenzae* lysogens to form plaques on a large number of indicator strains suggests that these phages were probably genetically defective. It is unlikely that the lack of plaque formation was due to the absence of suitable indicators or exclusion by restriction enzymes since some of the strains used for plating did not possess detectable restriction enzymes. Only one defective phage recovered from *H.influenzae* Rd was previously characterized morphologically (Boling *et al.*, 1973; Stachura *et al.*, 1969). This phage belongs to Group A1. Stachura *et al.*, (1969) attributed the lack of plaque-forming ability of this phage to the presence of incomplete particles. Although incomplete phage particles were also present in our phage preparations we did not establish whether the apparent absence of DNA in the phage heads was based on a genetic defect or was due to instability of the phage particles. Preliminary examination of

the ability of 2 phages (J1 and J9) to adsorb to homolgous lysogens cells showed that they failed to attach to the cells. However, we were not able to cure the strains of their prophages in order to test adsorption to homolgous nonlysogenic cells. Only one out of 19 lysogenic strains of *H.influenzae* described by Stuy (1978) released a plaque-forming phage while the remaining strains carried defective prophages. The finding that most phages recovered from *H.influenzae* isolates were unable to produce plaques and behaved as genetically defective, raises questions on their nature and origin. It appears unlikely that these phages arose from temperate bacteriophages by a mutation. It would be difficult to explain why strains carrying wild-type temperate bacteriophages occur in nature with much lower frequency than strains harbouring defective bacteriophages. Studies on other bacterial systems do not provide evidence that defective lysogenic strains have selective advantage over strains lysogenic to wild-type temperate bacteriophages. An alternative hypotheses regarding the origin of the phages described here is that they represent biological forms that are more primitive than temperate phages. The possible existence of such phages was first suggested by Seaman *et al.,* (1964) in order to explain the properties of inducible phages of *Bacillus subtilis* that were able to kill sensitive cells but due to their inability to replicate did not form plaques on the indicator strains. The authors proposed the term protophage to describe such an entity. The phages recovered from *H.influenzae* isolates are similar to those described by Seaman *et al.*, with respect to their inability to form plaques and the possession of killing properties. However, we have not yet studied the possibility that the *H.influenzae* phages described here may fail to undergo vegetative replication.

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