

STUDIES ON C. MEDIOLANUM R18 AND ITS PHAGE

**THESIS SUBMITTED
UNIVERSITY OF BOMBAY**

**FOR THE DEGREE OF
MASTER OF SCIENCE
IN
MICROBIOLOGY**

**SUBMITTED BY
SADHNA M. MOHILE**

**UNDER THE GUIDANCE OF
DR. S. V. GADRE**

**HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
BOMBAY**

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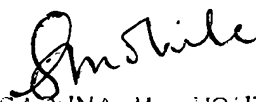
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(SADHNA M. MOHILE)

Candidate

Forwarded through the Research Guide -


(DR. S. V. GADRE)

Research Guide



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INTRODUCTION

In almost every civilization, primitive or advanced, man has practised the fermentation of fruit, grain or milk to obtain intoxicating and nourishing dietary factors. Evidence of wine production as early as 3000 B.C. has been found in excavations at Lachish and other sites (Wooley, 1965). The systematic application of these early techniques could come only after the scientific practice of organic chemistry and microbiology began in the 19th century, when the concept that individual microbial species were responsible for the discrete alterations of selected substrates was elucidated for the first time (Charney and Herzog, 1967). The significant advances made in microbiology have revealed the nature of these chemical processes, leading not only to great improvements in many of them, but also to the development of entirely new pharmaceutical industries which use microorganisms for the production of amino acids, antibiotics, vitamins, enzymes and transformation of steroids.

Steroids are physiologically active compounds used mainly as immuno-suppressors in the treatment of autoimmune diseases, allergies and hormonal imbalances. A steroid molecule is rather complex in its chemical

structure and is based on the "cyclopentanoperhydro-phenanthrene" nucleus. Specific chemical groups at vital positions on the molecule, however, determine the physiological activity of the steroid molecule. The alterations of these groups on the molecule can be effectively manouvered by exploiting the specificity and efficiency of the microbial enzymes.

The first patented process for the microbial transformation of steroids was developed by Mamoli and Vercellone (1938). Through the use of impure yeast cultures they discovered a class of sequential oxidation-isomerisation reactions which they later correctly attributed to the action of bacterial contaminants. Mamoli (1939) separated a pure culture of yellow colonies from the bacterial contaminants and identified it as Corynebacterium helvolum, which is now known as Corynebacterium mediolanum. This culture is now employed by several laboratories in the world to bring about the conversion of 3β ,21-dihydroxy-5-pregnen-20-one-21-acetate to deoxycorticosterone. In order to bring about this type of conversion in a fermentor, the sterilized growth medium is inoculated with the culture. As soon as the culture reaches a critical cell mass, in a specific phase of growth, the steroid is 'charged' into the fermentor for conversion.

The process of conversion of biologically important

molecules by growing bacteria can be affected by physical, chemical and biological conditions prevailing in the fermentation tank viz., the pH and oxygen tension of the medium, the levels of the different chemical constituents of the medium and the presence of certain biological inhibitors such as a bacterial virus (bacteriophage) or bacteriocins.

Bacteriophages (phages, in short), are bacterial viruses, that attack and kill bacteria. They are ultra-microscopic in size and possess a very simple structure which consists of DNA or RNA as their genome protected by a protein coat. They are not capable of multiplying by themselves, but use the replicating machinery of a susceptible actively growing host and are thus, totally parasitic in nature.

Invasion of the bacterial host by a phage involves the following steps :

- 1) Adsorption of the phage to a specific receptor on the host.
- 2) Penetration of the phage nucleic acid into the host.
- 3) Replication of the phage nucleic acid and synthesis of phage coat proteins, and finally,
- 4) Maturation of the phage followed by the lysis of the host.

Phages were discovered independently by Twort (1915) and d'Herelle (1917) but remained a laboratory curiosity for a long time afterwards. They owe their present day status as a model tool in biological research, largely to the stimulus provided by pioneers like Burnet (1929), Ellis and Delbruck (1939), Schlesinger (1932-1934), Luria and Latarjet (1947), Hershey and Chase (1952) in introducing and exploiting improved methods of experimental analysis.

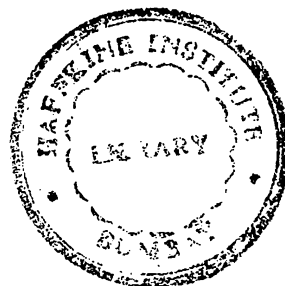
d'Herelle (1926) in his book "The Bacteriophage and its Behaviour" presented the method for quantitative determination of phages viz., the plaque method, which is the predecessor of the present day plaque assay. Burnet (1929) devised the "single burst experiment" to determine the phage yields from single cells. Ellis and Delbruck (1939) designed the classical "one step growth experiment" which could detect the latent period and average burst size of the phage. The one step growth curve and single burst experiments were primarily a means to explore the nature of phage behaviour and its replication within the cell.

Schlesinger (1932a, 1932b, 1932c, 1933a, 1933b, 1934) has presented important information about the dimensions of phage particles using a method of centrifugation and ultrafiltration. This method also allowed him

to purify sufficient quantities of phage for chemical analysis. He showed further that, his pure phage material consisted of approximately equal amounts of protein and DNA. In 1940, it was possible to obtain visual impressions of viruses with the help of electron microscope (Ruska, 1940; Pfankuch and Kausche, 1940).

Luria (1947) studied the effect of radiation on phage particles. Luria and Latarjet (1947) provided some information about the mechanism of intracellular phage multiplication through irradiation of infected cells. Hershey and Chase (1952) provided convincing evidence of penetration of phage DNA into the host using the T2 coliphage labelled with radioactive phosphorus and sulfur. Their experiment showed that while infecting a bacterial cell, the entire phage particle does not enter the cell but that the protein coat is left outside while only the phage genome penetrates into the cell.

All the initial work was done on the T series of phages and their host E. coli. These phages eventually became the chosen tool in the examination of genetic processes leading to an understanding of the nature of bacterial viruses and ultimately to the cracking of the genetic code.



The extent of damage to a fermentation process due to a phage attack depends on the phase of the fermentation during which the bacteriophage gains entry into the fermentor. If the phage finds an actively growing susceptible organism mediating a fermentation, it will infect and lyse those cells, bringing about an abrupt halt to that fermentation. Such an incidence results in heavy losses to the industry in terms of destruction of raw material as well as the time and money spent on ensuring that the fermentation equipment is treated carefully to get rid of the phage before a new batch of the substrate is charged. In contrast, an infection occurring late in the fermentation process will hardly be noticed since most of the substrate conversion is already complete and the host cells have entered the stationary phase which does not permit efficient phage growth.

Many instances where a fermentation has suffered due to a phage attack are known. In dairy industry, a decline in the rate of fermentation by lactic acid producing streptococci due to lysis of the organisms by a phage has been reported by Sanders and Klaen-Hammer (1983); interference in acetone-butanol fermentation by Clostridium acetobutylicum due to a phage attack (Casida, 1968) are but a few instances quoted here.

A pharmaceutical concern in Bombay which carries out transformation of steroids uses several bacterial and fungal cultures in their plant for the conversions; Corynebacterium mediolanum being one of the bacterial cultures used. The R and D laboratory of the industry isolated a bacteriophage which could attack this culture. The phage in this case was termed as phage 39. Since this phage 39 posed a threat to the desired conversion, this company was interested in developing means to stop this interference by the phage.

There are several means to prevent the phage from attacking the bacterial host in a fermentor. Heating or filtering of the air fed into the fermentation vessel to prevent the entry of the phage has not been proved to be a fool-proof method and is not employed in most of the pharmaceuticals including the one mentioned above.

Two other methods exist for combating the phage inside the fermentation vessel. The first method involves use of specific bacteriophage inhibitors in the fermentation medium so that it cannot attack the host i.e., the bacterial cell. This could ensure uninterrupted growth of the host and the desired enzyme activities, but the inhibitor employed could interfere with the normal metabolism of the bacterial cell or with the recovery of the transformed product. The second method is the

isolation of stable bacteriophage resistant mutants of the bacterial culture.

Hence, this study began with the development of stable phage resistant mutants of C. mediolanum against phage 39.

Standard methods are available to develop mutant bacteria, i.e., bacteria with a change in the reading frame of certain specific genes in their genome, thereby causing a phenotypic change expressed through the altered products coded by these genes. The genes responsible for phage resistance are those which lead to interference with any one of steps in the lytic cycle of the bacteriophage viz., adsorption, penetration, replication and maturation of the phage.

Microbial populations are remarkably homogeneous. A population of 10^9 bacteria contains very low percentage of genetic variation. However, spontaneous variation does occur, albeit at a very low rate, due to occasional 'errors' inherent in the process of DNA replication that escapes the proof reading, editing and repair mechanisms that are responsible for maintaining the fidelity of the system. These mutants can be isolated by growing a population of bacteria in the presence of manifold units of the phage. The whole process can be speeded up quite considerably (10 to 1,00,000 fold) by

using mutagenic agents to increase the percentage of mutants in the population.

There are two main categories of mutagens viz., physical and chemical. Physical mutagenesis can be carried out through irradiations using either ionizing radiations (X-rays, γ -rays, neutrons, etc.), or non-ionizing radiations (ultraviolet radiation), while, chemical mutagenesis can be carried out by the vast array of mutagens available. These include base analogues (2-amino purine, 5-bromo uracil), nitrous acid, hydroxylamine, acriflavines, alkylating agents (ethyl-methane sulphonate, methyl-methane sulphonate and N-methyl-N'-nitro-N'-nitrosoguanidine).

This particular pharmaceutical industry in Bombay uses three different species of Flavobacterium dehydrogenans, Corynebacterium simplex, and Corynebacterium mediolanum. These organisms have the capacity to transform a steroid molecule through the action of several enzymes such as dehydrogenases, oxidases and esterases that they produce. Almost all these cultures play host to their respective phages.

In a earlier study in our laboratory (Dandekar, 1984), it was observed that the cultures employed by the company to bring about various conversions share some of

the enzymes and are also susceptible to certain common phages indicating a close genetic and biochemical relationship between these cultures. This relationship could be better understood through characterization of the phages and the hosts. Dandekar (1984) has characterized the phages of C. simplex and F. dehydrogenans.

The present study involved :

- 1) Development of phage resistant mutants with desired enzymatic activities, and
- 2) Characterization of the phage and its nucleic acid.

LITERATURE REVIEW

LITERATURE REVIEW

This is the first report on C. mediolanum and its phage system. The literature relevant to the techniques used for studying the phage and the phage resistant mutants, has therefore, been reviewed in this chapter.

2.1 Isolation of Mutants :

Every bacterium, in the course of its multiplication is subjected to spontaneous variation arising from the change(s) in sequence of bases in the DNA (deoxyribonucleic acid), the genetic material of the living cell. The rate of mutation can be increased considerably by employing physical or chemical agents.

2.1.1 Natural Selection :

Natural selection is in fact, the selection of mutants that have been generated spontaneously in a population of sensitive cells. This can be achieved by incubating actively growing bacterial cells with appropriate selective agents. Phage resistant mutants can thus be isolated by growing the host cells in presence of manifold units of the phage and selecting out the resistant mutants that survive (Adams, 1959).

2.1.2 Physical mutagenesis :

Nucleic acids are known for their ability to absorb light rays of different wavelengths. Ionizing radiations such as X-rays, γ -rays, neutrons, etc. are known to interact with the nucleic acids. However, these radiations induce profound structural changes like transversions and inversions in the chromosome of survivors leading to loss of certain important properties of the bacterial species, and at times even chromosomal breakages, leading to high rate of inactivation of cells (Hopwood, 1970). Hence, these radiations cannot be used to bring about precise alterations in the host cell.

U.V.radiation is generally preferred to ionizing radiations on two grounds : 1) Ratio of mutation induction to lethal effect is high, 2) Mutagenic action of U.V.radiation is easily containable (Beckeriche et al., 1984).

U.V.radiation induces the formation of pyrimidine dimers, i.e., creation of covalent linkage between adjacent thymine or cytosine residues on the same DNA strand. These pyrimidine dimers are lethal unless they are repaired.

U.V. light is also known to bring about the hydration of the C₅-C₆ double bond of the pyrimidines to

form 5-hydro-6-hydroxy derivatives. Some cross linking may occur probably involving pyrimidines between complementary chains of double stranded nucleic acid (Knight, 1975).

A number of repair mechanisms, such as photo-reactivation and dark repair, have been identified in E. coli. Most of these are "error-free" mechanisms which restore the original DNA sequence with a efficiency comparable to that of normal methods of DNA replication. Mutations arise from the operation of an "error prone" repair pathway, through which the chances of incorrect bases being inserted are considerably increased (Dale, 1985).

The mutagenic wavelength of U.V. lies between 200-300 nm, the peak of absorption being 260 nm. Low pressure mercury vapour lamp emits a very high proportion of its energy at 254 nm, close to the most effective wavelength (Meynell and Meynell, 1965).

2.1.3 Chemical Mutagenesis :

A vast array of chemical mutagens are available, but few are convenient for routine isolation of mutants. Amongst these are the base analogues like 2-amino purine and 5-bromo uracil which substitute for the natural bases, nitrous acid which deaminates the purine and pyrimidine

residues of the DNA, acridine orange, acriflavin and ethidium bromide which intercalate between the stacked bases of the DNA strands and alkylating agents like Ethyl-methane sulphonate, Methyl-methane sulphonate, N-methyl-N'-nitro-N'-nitrosoguanidine which alkylate certain ring nitrogen atoms of purine and pyrimidine bases (Stanier et al., 1980).

Alkylating agents are more efficient than the base analogs or agents such as nitrous acid or hydroxylamine in inducing mutations. However, because of the variety of cellular constituents subject to attack and the variety of reaction products generated, the specific mutagenic lesions induced by these agents are difficult to identify (Jacobson, 1981).

NTG is the preferred mutagen because of its high mutation to lethality ratio. It interacts with the replication fork in replicating DNA causing base pair substitutions at the replicating fork (Kimball and Setlow, 1974).

NTG belongs to a class of mutagens that form methylated bases in DNA. NTG is a monofunctional alkylator i.e., it donates only one alkyl group causing alkylation of adenine and guanine. In addition to producing 7-methyl guanine and 3-methyl adenine adducts, NTG

also produces methylation of guanine at O⁶ position (m⁶G) in a considerably high proportion of guanine molecules (i.e., 7%). The m⁶G residues are responsible for the high mutagenic activity of NTG (Luchessi et al., 1986). These m⁶G residues in replicating DNA can pair with either cytosine or thymine; pairing with thymine should induce GC to AT transition mutations (Luchessi et al., 1986). Analysis of NTG induced nonsense mutations in the lac I gene of E. coli supports this view (Coulondre and Miller, 1977).

NTG has been successfully used for developing phage resistant mutant strains of industrially important bacterial species. Yu et al. (1986) have used NTG for the isolation of phage resistant mutants of Bacillus subtilis culture used for α -amylase production.

2.2 Mechanism of Resistance to Phage:

As mentioned earlier, the phage lytic cycle involves the following steps - 1) adsorption of phage to specific receptors on the cell surface of the host, 2) penetration of the phage nucleic acid, 3) replication of phage nucleic acid and synthesis of phage coat proteins and 4) maturation of phage followed by lysis of host releasing phage progeny.

On entering the host cell, in addition to initiating its own replication, the phage nucleic acid directs the

synthesis of endonucleases which specifically destroy the host DNA, leading to gross disruption of the host cell metabolism. Interference with the adsorption of the phage particle onto the host cell surface, penetration of the phage nucleic acid or the intracellular transcription of the phage nucleic acid can, therefore, effectively intervene the phage lytic cycle destined to disrupt the host cell metabolism.

2.3.1 Interference with the Adsorption of the Phage :

Adsorption is defined as the attachment of the phage particle to specific receptor moieties on the host cell surface.

These receptor moieties on the bacterial cell surface probably serve other useful purposes, such as iron transport across the host cell surface (Hantke and Braun, 1973). This observation is supported by several other workers.

Two flagellotropic phage PBS1 (which infects motile strains of Bacillus subtilis) and PBP1 (which infects motile strains of Bacillus pumilus) attach to their host by winding their tail fibres round the flagellar filament (Joys, 1965; Lovett, 1972).

Phage P22 of S. typhimurium uses 'O' antigen as its receptor (Israel et al., 1972), while phage T4 of

E. coli B uses the R-core of LPS as receptor (Préhm et al., 1976).

RNA phage Ø 6 attaches with its tail like structure to the pili of Pseudomonas phaseolicola (Bamford et al., 1976).

A regular array of protein that is present at the outer surface of Bacillus brevis strain P1 has been found to act as receptor to several phages (Sleytr, 1978). Randall (1980) has therefore put forward the suggestion that the receptor moieties present on the cell surface are for other useful purposes but have been usurped by the phages.

A mutation resulting in loss, alteration or masking of the cell wall receptors can lead to phage resistance.

The presence of hyaluronic acid capsule in streptococci can block access of phages to receptors in the underlying cell walls (Krause, 1957). The presence of microcapsular polysaccharide in Staphylococcus aureus has been shown to confer phage resistance on bacteria by blocking access to cell wall material (Wu and Park, 1971).

Similarly enhanced production of protein 'A' by Staphylococci and protein 'M' by Streptococci confers phage resistance (Nordstrom and Forsgren, 1974; Cleary and Johnson, 1977).

Bacterial mutants of E. coli devoid of flagella or bearing a minor structural defect in flagellin have been shown to be resistant to phage $\chi 1$ (Silverman and Simon, 1973; Icho and Iino, 1978).

2.3.2 Interference with the Penetration of Phage Nucleic Acid :

Not much is known about phage resistance at the level of penetration of nucleic acid. Scandella and Arber (1974) have isolated mutants of E. coli defective in the penetration of phage lambda (Pel^-) which permit irreversible adsorption of the phage but no penetration. It was observed by Luria and Steiner (1954) that adsorption of coliphage T5 onto host bacteria is not followed by full penetration of phage DNA unless Ca^{++} or Mg^{++} are present in the medium. Even in the presence of Ca^{++} the penetration of DNA is very slow and takes several minutes for completion, leading to partial resistance to the phage.

2.3.3 Interference with the Replication/Transcription of the Phage Nucleic Acid :

Chamberlin (1974) has isolated NTG mutants of E. coli B1 which adsorb the phage T7 normally but do not permit intracellular growth.

A mutant of E. coli Bol I hd 59 was found to be incapable of functioning as a host for T4 and T7 bacteriophages (Simon et al., 1974).

Interference at the intracellular level could be due to restriction of the phage nucleic acid in the host. Hattman and Fukasawa (1963) have reported a host induced modification system in T-even coliphages wherein an E. coli host lacking in the product uridine diphosphoglucose (which modifies the phage DNA) brings about restriction of the phage DNA by specific nucleases.

Restriction of the phage nucleic acid by the host is known to involve several other mechanisms resulting from specific mutations in the host. These mechanisms have been discussed later in the context of our observations in the present study (See Chapter V - Discussion).

2.4 Characterization of Phage and Its Nucleic Acid :

Bacteriophages are a diverse group of entities, having an enormous size range. They include tailed, cubic

filamentous and pleomorphic members. Further, their nucleic acid may consist of DNA or RNA in single stranded or double stranded form.

Primary attempt at characterisation of the phage is made usually on the basis of its nucleic acid character and gross particle morphology. Using these and other criteria phages are currently divided into ten major groups (Mathews, 1979), labelled A through G in continuation of an earlier system of nomenclature (Bradley, 1967).

Tailed phages are by far the most common type of phage occurring in over 90 genera of bacteria and cyanobacteria. Almost all tailed phages contain linear double stranded DNA (Reanney and Ackermann, 1982).

Tailed phages are subdivided into three basic groups on the basis of their tail structure.

Group A - Myoviridae : contractile tails.

Group B - Styloviridae : long non-contractile tails.

Group C - Podoviridae : short tails.

Phages with isometric heads and different tail structures as above (comprising of A₁, B₁ and C₁ morphotypes) constitute over 80% of known phages.

Following are some of the criteria for the characterization of viruses laid down by the International Committee on Taxonomy of Viruses (Ackermann et al., 1978):

- 1) Determination of the size and shape of the virus.
- 2) Determination of the type and structure of nucleic acid.
- 3) Determination of the molecular weight of the nucleic acid.
- 4) Latent period and burst size of the virus.

There are various other criteria based on the structural and physiological properties of the phage.

2.5 Purification of the Phage :

Among the various features used in a descriptive characterization of a phage are the biophysical and biochemical properties of the phage particles. Before these properties of the phage can be determined the particles have to be purified.

There are several methods currently in use for the purification of viruses of bacterial, animal or plant origin. These include the use of differential centrifugation, enzymatic treatment, extraction with organic solvents, precipitation with salts, adsorption to various

materials, partition in liquid two phase systems, electrophoresis and serological methods.

Precipitation with ammonium sulphate has been used to obtain highly purified crystalline preparations of several plant viruses such as southern bean mosaic, turnip yellow mosaic, squash mosaic, tomato bushy stunt and tobacco necrosis viruses (Price, 1946; Markham et al., 1948; Takahoshi, 1948; and Bawden, 1950).

Adsorption to magnesium pyrophosphate has been used to purify coliphages by Schito (1966).

Bacharach and Schwerdt (1952) have used n-butanol for the purification of poliomyelitis virus.

Albertsson (1971) described several two phase systems of water soluble polymers such as dextran-methyl cellulose, dextran-polyethylene glycol in which animal, plant and bacterial viruses could be selectively concentrated and at least partially purified. Concentration of a virus by this method is based on its low partition coefficient in systems used, which by proper adjustment of phase volumes permits concentration of most of the virus in a low phase (or interface).

A property that has been very helpful in the purification of some viruses is their resistance to attack

by proteolytic enzymes and nucleases; for example, Herriott and Barlow (1952) employed DNase treatment as a step in the purification of T2 bacteriophage in order to destroy host DNA which might otherwise adsorb to the phage.

The size of the most of the viruses permitted sedimentation of these viruses from solution in an hour or two in centrifugal fields of 40,000 to 100,000 g as early as 1959 (Stanley, 1959). Prior cycle of low speed centrifugation helped removal of the host cell debris. The method can be further improved by differential centrifugation which involves alternate cycles of low and high speed centrifugation in order to effectively remove the host cell wall material, denatured proteins, etc.

In density gradient centrifugation, the separation of particles takes place on the basis of their respective densities in a convection free medium. It can be achieved either by rate zonal density gradient centrifugation or by isopycnic density gradient centrifugation. Schwerdt and Schaffer (1956) used rate zonal density gradient centrifugation to purify poliovirus, while Sinsheimer (1959) used equilibrium density gradient centrifugation for the purification of ϕ x174 bacteriophage.

None of the methods as listed above when employed singly is capable of providing highly purified preparations of viruses by itself, but when used in combination these methods provide very pure preparations of viruses.

2.6 Extraction of Viral Nucleic Acid :

Nucleic acids are extracted from the virus by treatment with detergents, with salts at high temperature, by combination of the two, or by phenol, phenol-detergent, alkaline hydrolysis, guanidine hydrochloride, strontium nitrate treatment, etc.

A combination of salt and detergent has been used by Dorner and Knight (1953) to obtain good yields of nucleic acids. Sodium dodecyl sulphate (a detergent) has been used to extract RNA of tobacco mosaic virus by Frankael-Conrat et al. (1957). Guanidine hydrochloride has been used to extract 70-90% of infectious RNA by Riechmann and Stace-Smith (1959).

Extraction of the nucleic acid with salt at high temperature involves heating the virus in an aqueous salt solution followed by chilling in an ice-bath. The osmotic shock and sudden chilling causes the rupture of the protein coat releasing the nucleic acid which is separated with precipitation and centrifugation with ethanol. This method was used to extract DNA from phage ϕ x174 (Guthrie and Sinsheimer, 1960).

DNA is found to be fairly resistant to alkaline media upto pH 12 and this fact was used to isolate the nucleic acid of nuclear polyhedrosis virus of silkworm Bombyx mori (Onodera et al., 1965).

The phenol extraction method of Westphal et al. (1952) is perhaps the most common procedure used for obtaining the nucleic acid from a wide variety of viruses. In practice two layers - a phenolic layer and an aqueous layer are obtained and protein is extracted into the phenolic layer and some into the interface while nucleic acid and polysaccharide (if present) goes into the aqueous layer.

In some instances it has been found beneficial if the phenol extraction of nucleic acid is carried out after the virus has been opened by a detergent such as sodium dodecyl sulphate. Rushizky and Knight (1959) used such a method to obtain infectious RNA from tomato bushy stunt virus. Wahl et al. (1960) used this method to prepare ϕ X174 DNA. Maniatis et al. (1982) have suggested this method for rapid small scale isolation of phage λ DNA.

2.7 Determination of Nucleic Acid Character :

The nucleic acid character can be determined by any one of the following methods : Acridine orange staining,

alkaline denaturation, sensitivity to enzymes DNase and RNase and chemical determination of sugars.

Acridine orange staining was used by Bradley (1966) to distinguish between DNA and RNA of several phages. Russell (1962) used the diphenyl amine method of Burton (1956) specific for DNA and orcinol method of Dische (1955) specific for RNA to show that the nucleic acid of Herpes virus is of the deoxy type.

Rushizky and Knight (1959) showed that the nucleic acid of tomato bushy stunt virus was RNA by demonstrating sensitivity to enzyme ribonuclease.

2.8 Determination of the Structure of Nucleic Acid :

The number of strands in a nucleic acid can be determined either by acridine orange staining, or by chemical analysis of base ratio, determination of bouyant density and thermal denaturation profiles.

Acridine orange is a dye that can differentiate between double and single stranded nucleic acids irrespective of DNA or RNA. It can intercalate double stranded helical nucleic acids to give green fluorescence under U.V.light (Lerman, 1963) or bind electrostatically to phosphate groups of single stranded molecules to give red fluorescence under U.V.light (Bradley, 1959).

Gomatos et al. (1962) proved that Reo virus contains a double stranded RNA by staining cytoplasmic inclusions with acridine orange. They observed that cytoplasmic inclusions in Type 3 infected *L* cells stained pale green with acridine orange. At a pH of 5.6 double stranded DNA or RNA binds a small amount of the dye and fluoresces greenish yellow as compared to single stranded DNA and RNA which have higher binding capacity and stain bright red.

The chemically determined base composition of the single stranded DNA from small bacteriophages like ϕ X174, fd, M13 deviate widely from those predicted for a complementary structure i.e., double stranded DNA where, A = T and G = C. The base thymine accounts for about one-third of the bases in the DNA of all the above ssDNA bacteriophages. The observed non-complementary base ratios are consistent with all the DNA's being single stranded (Ray, 1968).

Gierer (1960) found that TMV-RNA could be heated at 70°C for 10 minutes without any rise in absorbance indicating that there was no denaturation of DNA. Since this heat treatment is known to disrupt hydrogen bonds it was concluded that TMV RNA was a single large polynucleotide strand.

Pina and Green (1965) showed through bouyant density studies on adenovirus 2 that nucleic acid exhibited a molar equivalence of bases i.e., $A/T=G/C=1$ which is characteristic of ds DNA.

2.9 Determination of Molecular Weight of Nucleic Acid :

The molecular weight of a nucleic acid is usually determined by measuring the contour length of the nucleic acid from an electron micrograph. Mayer et al. (1974) determined the molecular weight of plasmid DNA isolated from Neisseria gonorrhoeae through measurement of its contour length from an electron microscopic photograph. The molecular weight can also be determined by subjecting the nucleic acid preparation to agarose gel electrophoresis using standard molecular weight markers (Schleif and Wensink, 1981).

The sedimentation coefficient of nucleic acid can also be used to determine its molecular weight. Anderer and Restle (1974) used this method to determine the molecular weight of the nucleic acid of poliomyelitis virus.

2.10 Determination of Purity and Concentration of Nucleic Acid :

Nucleic acids absorb U.V. light so efficiently that optical absorbance can be used as an accurate, rapid and non-destructive measure of their concentration even at

levels as low as 2.5 µg/ml. A very useful approximation of this standard is that double stranded RNA and DNA at 50 µg/ml in aqueous solution have an $A_{260 \text{ nm}}^{1 \text{ cm}}$ of 1 (Schleif and Wensink, 1981).

Absorbance is also a very useful measure of purity of the nucleic acid preparations. The absorbance spectrum of double and single stranded structures and the increase in absorbance during the transition from double stranded to single stranded forms is also a fairly accurate measure of purity.

The absorbance spectrum is usually the preferred measure of purity because it is more rapid and easily determined. It also does not require the denaturation of RNA or DNA. The relevant spectrum is between 230 nm and 320 nm. Absorbance at 320 nm which is more than several percent of the absorbance at 260 nm indicates the presence of undesirable foreign material. A ratio of $A_{260 \text{ nm}} / A_{280 \text{ nm}}$ should be between 1.65 to 1.85. Higher ratios often indicate RNA contamination and lower ratios indicate phenol or protein contamination (Schleif and Wensink, 1981).



MATERIALS & METHODS

MATERIALS AND METHODS

3.1 The Bacterial Strain :

Corynebacterium mediolanum R₁₈ strain was provided by the pharmaceutical industry.

3.2 The Bacteriophage :

The phage 39 of C. mediolanum R₁₈ was also provided by the industry.

3.3 Culture Media Employed in the Study :

The media used for growing and maintaining the above organism and its phage were F₁₈ broth and F₁₅ agar (See Appendix). The soft agar for the overlay medium was obtained by adding 0.8% agar to F₁₈ broth. The soft agar medium was dispensed in aliquots and sterilized.

These media were sterilized by autoclaving at 121°C for 15 min. After sterilization, the agar medium was cooled to 45°C and plates were poured. After allowing the agar to set, the plates were incubated overnight at 37°C to ensure sterility and then transferred to the refrigerator till required.

3.4 Maintenance, Preservation and Assay of the Culture :

The above strain was maintained on F₁₅ agar slopes

and subcultured periodically. Whenever required, a loopful of the culture was inoculated into 10 ml of broth and incubated overnight at 32°C. Purity of the culture was checked by plating out on agar plates and also by examining under the microscope.

In all the experiments carried out by us, 3-4 hours old culture (hereafter referred to as log phase culture) was obtained by inoculating 0.05 ml of the overnight broth culture in 10.0 ml of fresh broth and incubating on a rotary shaker (180 r.p.m.) at 32°C.

The average number of viable host cells in a log-phase culture was determined by surface spreading 0.1 ml of several serial ten-fold dilutions of the culture on agar plates. The plates were incubated for 36 hours at 32°C. Plates showing colonies between 30 and 300 were chosen for counting. From these counts the number of viable cells in a log-phase culture was calculated. It was found to be in the vicinity of 3×10^8 cfu/ml.

3.5 Maintenance of Phage :

Phage 39 was maintained in broth at 4°C. Chloroform was added to the phage suspension to prevent bacterial contamination.

3.6 Bacteriophage Assay :

The phage 39 titre was determined by the standard method of Adams (1959) using the agar overlay method of Gratia (1936). The phage suspension was subjected to serial ten-fold dilutions in F_{18} broth. To a series of molten cooled soft agar tubes, 0.1 ml of log phase host cell suspension (See Section 3.4) and 0.1 ml of appropriate phage dilutions were added. The above mixtures were then overlaid on sterile agar plates and allowed to solidify. The plates were then incubated overnight at 32°C . The next day the plates were observed for plaques produced on a lawn of the sensitive host strain. Each plaque is normally produced by the initial infection of a single bacterium with a single phage particle. Number of plaques on the above plates were counted and the titre of the original phage suspension was calculated. This was expressed in forms of plaque forming units (PFU) per ml.

3.7 Preparation of High Titre Phage :

Two different methods were used to prepare high titre phage stocks. These were the agar plate method (Adams, 1959) and broth method (Billing, 1969).

In the agar layer method 0.1 ml of log phase culture of C. mediolanum R_{18} (approximately 3×10^8 cells/ml) and 0.1 ml of phage 39 ($3 \times 10^5 - 3 \times 10^6$ PFU/ml) were mixed in 3 ml of molten cooled soft agar and overlaid on

the basal layer of the agar plate. The plates were then incubated overnight at 37°C and examined for confluent lysis. The overlaid soft agar was scrapped off from the plate and collected in a flask containing broth. The volume of broth taken in the flask was 5.0 ml per plate. The flask containing pooled phage was shaken for 30 mins on a rotary shaker to allow complete diffusion of phage into the broth from the soft agar. The mixture was then subjected to centrifugation at 5000 r.p.m. The supernatant containing the phage was separated aseptically and stored over chloroform at 4°C.

In broth method 10.0 ml of phage 39 suspension ($3 \times 10^8 - 3 \times 10^9$ PFU/ml) was mixed with 100.0 ml of log phase culture of C. mediolanum R₁₈. The flask was further incubated for 4 hours at 32°C on a rotary shaker. At this juncture total lysis of the host cells occurred which was indicated by the clearing of the host cell suspension. To this high titre phage suspension 10 ml of chloroform was added and the phage stock was 4°C for future use.

The yield of phage obtained through both the methods was $3 \times 10^{10} - 3 \times 10^{11}$ PFU/ml.

Development of Phage Resistant Mutants :

In all three different methods were employed to

develop phage resistant mutants. They were -

- 1) Natural selection (Adams, 1959).
- 2) Physical mutagenesis (Clowes and Hayes, 1968).
- 3) Chemical mutagenesis (Hopwood, 1970).

3.8 Natural Selection :

In this method log phase culture of C. mediolanum R₁₈ was inoculated with phage 39 (m.o.i. of $10^2 - 10^3$) and incubated on a shaker at 32°C. After 18 hours lysis was observed; however, on further incubation turbidity was observed presumably due to growth of naturally resistant mutants present amongst the population of sensitive cells. These naturally resistant mutants were isolated by streaking a loopful of the suspension on an agar plate. The resistance of the isolates to the phage was confirmed subsequently as given in Section 3.11 .

3.9 Physical Mutagenesis :

U.V. radiation is an extremely convenient mutagen since its mutagenic wavelength lies between 200-300 nm. The peak of absorption of nucleic acids is at 260 nm. The chances of obtaining the desired mutations are more when a U.V. dose that kills more than 99.9% of the cells is used. To ascertain this it was necessary to plot the survival curve of the host against exposure in seconds to U.V. irradiation.

3.9.1 Survival Curve :

Hundred ml of log phase culture of C. mediolanum R₁₈ was centrifuged at 5000 r.p.m. for 30 mins. The pellet was washed twice with phosphate buffered saline (PBS) and resuspended in 100.0 ml of the same menstrum. This was then dispensed in 7 ml aliquots in each of the ten sterile petri dishes (10 cm diameter) and subjected to U.V. radiation for 0, 30, 60, 90, 120, 150, 180, 210, 240 and 270 secs using a Philips TUV 15W G15 T8 U.V. lamp placed at a distance of 50 cms. The plate at 0 secs exposure was treated as control. The plates were then wrapped in black paper and kept in the dark for 45 mins to prevent photoreactivation. The cell suspensions were subjected to serial ten-fold dilutions and 0.1 ml of the appropriate dilutions were surface spread on agar plates. The plates were incubated for 36-48 hrs. The number of colonies were counted after the end of the incubation period and the viable count of each exposure was determined. This experiment was carried out in triplicate and a graph of the log₁₀ of mean number of survivors against the U.V. exposure in seconds was plotted.

3.9.2 Mutagenesis :

Fifty ml of log phase culture of C. mediolanum R₁₈ was centrifuged at 5000 r.p.m. for 30 mins. The pellet was washed twice with PBS and resuspended in the

same menstrum. This suspension was dispensed in 7 ml aliquots in four sterile petridishes and exposed to U.V.radiation for 150, 180, 210, 240 secs respectively. These plates were wrapped in black paper and kept in the dark to prevent photoreactivation.

After 45 mins these cell suspensions were centrifuged at 5000 r.p.m. for 30 mins and the pellet was resuspended in 7 ml of fresh broth. The tubes were incubated overnight at 32°C on the shaker for the segregation of mutant and non-mutant nuclei from the multinucleate cells and to allow the phenotypic expression of the induced mutation. After segregation, the mutagenized culture was plated on agar for obtaining isolated colonies which were to be tested for phage resistance.

3.10 Chemical Mutagenesis :

N-Methyl,N'-nitro,N'-nitrosoguanidine (NTG) is known as one of the most potent mutagens producing extremely high mutant yields under the favourable conditions.

3.10.1 Survival Curve :

Fifty ml of log phase culture of C. mediolanum R₁₈ was centrifuged at 5000 r.p.m. for 30 mins, washed twice with PBS and resuspended in 50 ml of the menstrum.

To this cell suspension NTG (1 mg/ml) was added in a series of test tubes to give final concentrations of 20, 40, 60, 80, 100 and 120 $\mu\text{g/ml}$. A tube without the addition of NTG served as control. The tubes were shaken at 32°C for 30 mins and then centrifuged at 5000 r.p.m. for 30 mins. The cells were washed twice with PBS to remove the NTG and resuspended in the required volume of PBS. Each tube was then subjected to serial ten-fold dilutions and 0.1 ml of the appropriate dilutions were surface spread on agar plates. The plates were incubated at 36-48 hrs at 32°C and the number of colonies that developed were counted. From these counts, the number of survivors for each concentration of NTG were calculated. This experiment was performed in triplicate and a graph of $\log_{10}$ of mean number of survivors against NTG dose was plotted.

3.10.2 Mutagenesis :

Fifty ml of log phase culture of C. mediolanum R₁₈ was centrifuged at 5000 r.p.m. and washed twice with PBS. The cells were then suspended in 20 ml of the menstium. To the cell suspension NTG (1 mg/ml) was added in a series of tubes to give final concentration of 40, 60, 80 and 100 $\mu\text{g/ml}$, respectively. The tubes were shaken at 32°C for 30 mins and then centrifuged at 5000 r.p.m. for 30 mins. The pellets were suspended in fresh broth

and incubated for 18 hrs at 32°C on a shaker for the segregation of mutant and non-mutant nuclei from the multinucleate cells and to permit the phenotypic expression of the induced mutations. After segregation, the mutagenised cultures were plated on agar plates for obtaining isolated colonies which were tested for phage resistance.

3.11 Testing for Phage Resistance :

Log phase cultures of the mutants isolated were prepared as given in Section 3.4. Two drops (0.1 ml) of the log phase culture was mixed with 3 ml of molten cooled soft agar and overlaid on a basal agar layer. A sterile nichrome wire was dipped in phage 39 lysate, and the overlaid agar was pricked with it. The plates were incubated at 32°C for 18 hrs. The next day the plate was checked for the presence of plaques surrounding the prick. A plate of C. mediolanum R₁₈ pricked with phage 39 served as a negative control.

II. Characterization of Phage 39 and Its Nucleic Acid :

3.12 Purification of Phage 39 :

Ten 500 ml flasks each containing 100 ml of broth were inoculated with 0.5 ml of overnight grown culture of C. mediolanum R₁₈. The flasks were then

incubated on a shaker at 32°C for 3 hrs. Each flask was then inoculated with 10.0 ml of phage suspension (1×10^8 PFU/ml), and further incubated on the shaker for 4 hrs. After 4 hrs there was total lysis in the broth. All the phage lysates were pooled and the titre was determined. It was found to be 2×10^{10} PFU/ml. Concentration and purification of the phage was carried out by a combination of different methods described by Maniatis et al. (1982).

One litre phage suspension prepared as described above, was treated with pancreatic DNase I and RNase A (at 1 µg/ml, see Appendix). The suspension was incubated at 37°C for 1 hr. This treatment digests the nucleic acids liberated by the lysed bacterial cells. The phage suspension was then centrifuged in a JA 21 Beckman centrifuge at 12,000 g for 20 mins at 4°C using JA 14 rotor to remove the cell debris. The supernatant was then collected in a 2 litre flask. To this 55.4 gms of sodium chloride was added (to have a final concentration of 1M), followed by the addition of 100 gms of polyethylene glycol (PEG 6000). After homogenizing the mixture by slow stirring on a magnetic stirrer at 25°C, the suspension was allowed to stand for two hours between 0-4°C in ice-water to facilitate precipitation of phage particles.

The precipitated phage particles were separated by centrifuging the suspension at 12,000 g for 20 mins, at 4°C in a JA 14 rotor. The pellet was gently resuspended in 16.0 ml of SM buffer. An equal volume of distilled chloroform was added to it and the mixture was vortexed for 30 secs. The PEG precipitated out. The aqueous phase (containing the phage particles) and the organic phase (chloroform) were separated at 1,600 g for 15 mins at 4°C in a JA 20 Beckman rotor. The precipitation of PEG was repeated twice followed by centrifugation at 1,600 g for 15 mins at 4°C each time. The aqueous phase was recovered and subjected to ultracentrifugation at 40,000 r.p.m. (1,35,000 g) for two hours at 4°C in a L5KB Beckman preparative ultracentrifuge using a Ti75 rotor. The bluish white precipitate of the phage was resuspended in 1.0 ml of SM buffer. This purified phage solution was stored at 0-4°C. The titre of this concentrated phage was ascertained to be 6×10^{12} PFU/ml.

3.13 Extraction of Phage Nucleic Acid :

The method used was that described by Maniatis et al. (1982). In all the steps described below siliconized Eppendorf tubes (see Appendix) were used for treating the phage and its nucleic acid.

To 1.0 ml of phage suspension 50 µg of proteinase K, 0.025 ml of 20% sodium dodecyl sulphate solution and

0.04 ml of 0.5 M EDTA (pH 8) was added. The mixture was incubated at 68°C for 15 mins and sequentially extracted with equal volumes of phenol, phenol:chloroform (1:1) and chloroform. The phage proteins precipitated at different steps were separated by centrifugation in a Heraeus hemofuge at 15,500 g for 15 mins. To the final aqueous phase an equal volume of distilled isopropanol was added. The contents were mixed well and stored at -70°C for 20 mins to facilitate the precipitation of phage nucleic acid. After 20 mins the contents were thawed and centrifuged in a hemofuge at 15,500 g for 15 mins at 4°C. The nucleic acid pellet was washed with 70% ethanol, dried and resuspended in 1 ml of TE buffer.

3.14 Determination of the Nature of Phage Nucleic Acid :

The method used was agarose gel electrophoresis employed by Aaij and Borst (1972) with some modifications.

3.14.1 Preparation of the Sample :

The samples to be subjected to electrophoresis were as follows :-

Well No. 1 : Phage nucleic acid.

Well No. 2 : Phage nucleic acid digested with DNase.

Well No. 3 : Phage nucleic acid digested with RNase.

Well No. 4 : Standard pBR 322 DNA.
Well No. 5 : pBR 322 digested with DNase I.
Well No. 6 : Standard yeast RNA.
Well No. 7 : Yeast RNA digested with RNase A.

Phage nucleic acid was prepared as given in
Section 3.13.

Ten μ l of phage nucleic acid was subjected to digestion with 0.05 μ g of DNase I at 37°C for 30 mins. Similarly 10 μ l of phage nucleic acid was digested with 0.05 μ g of RNase A at 37°C for 30 mins. pBR 322 DNA was also subjected to digestion with 0.05 μ g of DNase I at 37°C for 30 mins. Yeast RNA was digested with RNase A at 37°C for 30 mins.

3.14.2 Preparation of the Gel :

Agarose gel electrophoresis was carried out using 0.8 % agarose (Fluka) prepared in Tris Acetate Buffer (TAE) (pH 8) in a mini gel chamber of dimension 17 cms x 6 cms x 6 cms.

0.24 g of agarose was added to 30 ml of TAE buffer in a 100 ml flask and heated till the agarose dissolved. It was then cooled to about 50°C. The open ends of the mini gel chamber were closed using side locks. The edges of the gel were sealed with a small

quantity of agarose using a Pasteur pipette. When the seal had set the rest of the agarose was poured into the gel mould and immediately the comb was placed 1 cm from the cathode end of the gel to form sample wells taking care to place the comb in a line parallel to the edge, about 0.5 to 1 mm above the bottom of the gel chamber. The agarose was allowed to set completely. The side locks were carefully removed and 210 ml of TAE buffer (pH 8.0) was added gently into the troughs of the chamber so that the level of the buffer was about 3-4 mm above the surface of the gel.

3.14.3 Loading the Sample :

Five μ l of each sample was mixed with 5 μ l of the gel loading buffer (see Appendix) and the samples were loaded into the respective wells as mentioned in Section 3.14.1.

3.14.4 Migration of the Nucleic Acids :

Electrophoresis was carried out at a current of 40 mA and 5 V/cm for 3-3 $\frac{1}{2}$ hrs, till the dye in the loading buffer reached the anode end of the gel bed.

3.14.5 Staining the Gel :

The gel was immersed in a solution of 0.5 μ g/ml of ethidium bromide (EtBr) for 45 mins, so that the EtBr intercalated between the stacked bases of the nucleic acids.

The gel was then destained in 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ for 1 hr to eliminate the unbound EtBr so as to avoid background fluorescence when subjected to U.V. illumination.

3.14.6 Detection of the Nucleic Acid using a U.V. Source :

The EtBr-gel was then subjected to U.V. light of wavelength 250 nm. The nucleic acids show up as sharp orange red bands since EtBr gives out orange red fluorescence under U.V. light.

3.15 Determination of the Structure of Phage Nucleic Acid :

The method used to determine whether the phage nucleic acid was double stranded or single stranded was that of McMaster and Carmichael (1977).

0.8% agarose gel was prepared as described in Section 3.14.2 using 10 mM sodium phosphate buffer (pH 7). The samples to be loaded on the gel were as follows :-

Well No. 1 : Phage 39 nucleic acid.

Well No. 2 : Standard d.s. DNA (pBR 322 DNA).

Five μl of each sample was mixed with gel loading buffer and loaded into the gel wells in the order specified above. The electrophoresis was carried out as described in Section 3.14.4. The gel was stained

with 30 $\mu\text{g/ml}$ of acridine orange for 30 minutes. The gel was then transferred to an enamel pan for destaining in 10 mM sodium phosphate buffer (pH 7.0) for 1 hr. Enamel absorbed acridine orange at faster rate and thereby reduced the time required to remove the higher background fluorescence of the gel. The nucleic acid bands were observed under U.V. illumination at 250 nm. Acridine orange dye distinguishes between double stranded and single stranded nucleic acids exhibiting red fluorescence with single stranded nucleic acids and green fluorescence with double stranded nucleic acids.

3.16 Determination of the Molecular Weight of Phage 39 Nucleic Acid :

The method used to determine the molecular weight of phage 39 nucleic acid was co-electrophoresis of phage 39 nucleic acid with standard molecular weight markers in an agarose gel (Schleif and Wensink, 1981). The 0.8% agarose gel was prepared as mentioned in Section 3.14.2. Five μl of phage nucleic acid was mixed with gel loading buffer and loaded into the well. Similarly, 5 μl of phage λ DNA Hind III digest (in TE buffer) was mixed with gel loading buffer and loaded into the well. Electrophoresis was carried out at 40 mA and 5 V/cm for 3-3 $\frac{1}{2}$ hrs. At the end of the run the gel was stained and destained as given in Section 3.14.5 and observed under U.V.illumination. The extent of migration of each

band of phage λ DNA Hind III digest and phage 39 nucleic acid was measured.

A graph of the distance travelled by each band against the standard molecular weights, was plotted. The graph showed a sigmoid curve. Hence, the 1st, 4th and 7th bands were subjected to correction by the method of Southern (1979) to correct the curvature obtained in the graph. A straight line was obtained on plotting a graph of standard molecular weights against $1/(m-m_0)$, wherein, 'm' is the distance travelled by each band and 'm₀' is the factor calculated to give the best fit to a straight line of the form :

$$L = K_1/(m-m_0) + K_2$$

Three points defined as per the molecular weight standards (The values of the standard molecular weights were as given by Daniel et al., 1983). L₁, L₂ and L₃ and corresponding mobilities m₁, m₂ and m₃ were chosen for the 1st, 4th and 7th bands of the phage λ DNA Hind III digest for the calculation of m₀.

A straight line through these points is defined by imposing the conditions that the slopes of the two lines should be the same. The slope of one of these lines is -

$$\frac{L_1 - L_2}{1/(m_1 - m_0) - 1/(m_2 - m_0)}$$

and another is

$$\frac{L_2 - L_3}{1/(m_2 - m_0) - 1/(m_3 - m_0)}$$

Since the two equations are equal, solving for m_0 gives

$$m_0 = \frac{[m_3(L_2 - L_3)(m_2 - m_1) - m_1(L_1 - L_2)(m_3 - m_2)]}{[L_2 - L_3)(m_2 - m_1) - (L_1 - L_2)(m_3 - m_2)]}$$

Rearrangement gives -

$$m_0 = \frac{m_3 - m_1}{1 - A}$$

where, $A = \frac{L_1 - L_2}{L_2 - L_3} \times \frac{m_3 - m_2}{m_2 - m_1}$

Hence the value of m_0 was determined and a graph of standard molecular weight against $1/(m - m_0)$ was plotted which gave a straight line. The molecular weight of the phage 39 nucleic acid was then determined by extrapolation of its value on the graph.

3.17 Determination of the Purity and Concentration of Phage 39 Nucleic Acid :

The purity and the concentration of the phage 39 nucleic acid was determined by the method of Schleif and Wensink (1981).

Standard calf thymus DNA (1 mg/ml) was prepared in TE buffer (pH 8.0). It was further diluted in buffer to give final concentrations of 20, 40, 60, 80 and 100 µg/ml. The absorbance of each concentration of DNA was determined at 260 nm in a Shimadzu U.V. spectrophotometer using TE buffer as blank to adjust zero reading.

Hundred µl of phage 39 nucleic acid was diluted 1:30 times and the absorbance spectrum was determined between 230 and 320 nm.

A graph of absorbance of the calf thymus DNA at 260 nm against known concentration of the phage 39 nucleic acid was determined by extrapolation of its absorbance at 260 nm on the standard graph.

The purity of the phage 39 nucleic acid was ascertained through the ratio of its absorbance at 260 nm and 280 nm (A_{260}/A_{280}) and also by the ratio of the absorbance of phage nucleic acid at 320 nm and 260 nm ($A_{320}/A_{260} \times 100$).

3.18 Generation of Antiphage 39 Antibody :

Rabbits were used for the production of phage 39 antiserum. One ml of the purified phage 39 suspension (6×10^{12} PFU/ml) (prepared as given in Section 3.12)

was inoculated intravenously, through marginal ear vein in rabbits weighing over 2.5 kgs, every week for a period of four weeks. The titre determined by the end of this period was found to be low and hence, a booster shot of 1 ml of phage 39 suspension was given.

One week thereafter blood was drawn by cardiac puncture. The serum was centrifuged at 2000 r.p.m. in a table top centrifuge to separate RBCs and inactivated by incubation in a serological water bath (56°C) for 30 mins. It was dispensed in aliquots and frozen at -20°C till required.

3.19 One step Growth Experiment : (Adams, 1959)

The one step growth experiment enables the determination of the latent period and average burst size of the phage.

3.19.1 Reagents :

1) Log phase culture of C. mediolanum R₁₈ diluted in broth to a concentration of 5×10^7 cells/ml.

2) Phage 39 diluted in broth to concentration of 5×10^7 PFU/ml.

3) Phage antiserum diluted to 1:50 in normal saline.

3.19.2 Protocol :

<u>Time in mins.</u>	<u>Tube</u>	<u>Procedure</u>
0	1 Adsorption	0.1 ml of diluted phage to 0.9 ml of cells with aeration.
5	2 Serum	0.1 ml of tube # 1 to 0.9 ml of 1:50 diluted antiserum.
10	3 Dilution	0.1 ml of tube # 2 to 0.9 ml of broth.
11	4 FGT	0.1 ml of tube # 3 to 3.9 ml of broth.
12	5 SGT	0.1 ml of tube # 4 to 9.9 ml of broth.

FGT is the first growth tube and SGT is the second growth tube.

Samples (0.1 ml) were drawn from tubes # 4 and 5 i.e., FGT and SGT starting from 30 mins upto 210 mins and were plated with 2-3 drops of the host culture by agar layer technique. The plates were incubated overnight. The next day the number of plaques in each plate were counted and the latent period and burst size calculated.

3.20 Single Burst Experiment : (Adams, 1959)

In this experiment, the infected bacteria are diluted to such an extent that an aliquot of the dilution

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contains one or less than one infected bacterium. The aliquots are incubated till all the bursts have taken place and the yield is determined by plaque assay.

3.20.1 Reagents :

1) Log phase culture of C. *mediolanum* R₁₈ diluted to 5×10^7 cells/ml.

2) Phage 39 diluted to 2×10^9 PFU/ml.

3) Phage 39 antiserum diluted 1:50 with normal saline.

3.20.2 Protocol :

<u>Time</u>	<u>Tube</u>	<u>Procedure</u>
0 mins	# Adsorption	0.1 ml of phage to 0.9 ml of culture.
5 mins	# Serum	0.1 ml of tube # 1 to 0.9 ml of 1:50 diluted antiserum.
10 mins	# 3 Dilutions	0.1 ml of # 2 to 9.9 ml of broth.
11 mins	# 4 Dilutions	0.1 ml of # 3 to 9.9 ml of broth.
12 mins	# 5 sample flask	0.1 ml of # 4 to 70 ml broth.

The broth suspension containing less than one infected bacteria/ml was distributed in 0.5 ml samples

into 40 tubes. The distribution was completed before the end of the latent period. The samples and the remainder of the suspension in the sample flask were incubated at 32°C till all the bursts had taken place. All the 40 samples were then plated with the host bacteria by agar layer technique. Several 0.5 ml samples from the sample flask containing the remainder of the diluted suspension were also plated to determine the average burst size in the suspension. The plates were incubated overnight at the end of which the number of plaques in each plate were counted. The burst size of the phage was calculated using Poissons distribution theory.

3.21 Electron Microscopy :

Electron micrographs of phage 39 were obtained with the kind help of Dr. A.N.Bhisey, Head, Department of Cell Biology, Cancer Research Institute, Parel, Bombay-12.

3.21.1 Sample Preparation :

The method used was that of Tikhonenko (1970) with few modifications.

The purified phage was obtained as given in Section 3.12.

3.21.2 Loading of the Sample :

The objects to be examined under the electron microscope were held in grids covered by a thin mounting

film. The role of the slide in case of electron microscope was performed by thin transparent film fixed to the grid. The grid was coated with formvar which was less than 200 Å in thickness.

3.21,3 Contrasting of the Objects : The structural details of the objects to be examined under the electron microscope can be revealed through negative staining of the objects using heavy salts such as uranyl acetate, uranyl oxalate, phosphotungstic acid as filling materials in which the object is sealed. Light particles of the object to be examined are arranged against a dark background of the contrasting material and its structural details are viewed. In present case the procedure adopted was as follows :

The formvar coated grid was coated with cytochrome 'C' (0.2 mg/ml in amyl alcohol) for 2 mins, the excess of cytochrome 'C' was removed by means of a filter paper strip and the grid was allowed to dry. Small drops of purified phage preparation were placed on the grids and left in that position for 3-5 mins in order to allow the phage to adsorb on the film. The excess of the sample was removed with the filter paper strip and the grids were allowed to dry. The grids were then inverted over drops of 0.5 % aqueous solution of uranyl oxalate for 5 mins. The excess of stain was removed by

means of filter paper and allowed to dry. The grid was then mounted on the electron microscope. The principle of negative staining favours the reliable preservation of shape and structure of the object because while it dries, the object is protected from the forces of surface tension by the material surrounding it. The photographs were taken at a magnification of 1,40,000 and the head and tail dimensions calculated.

III. Study of Resistant Isolates :

3.22 Determination of the Adsorption Rate of *C. mediolanum* R₁₈ and its Mutants :

Log phase culture of *C. mediolanum* R₁₈, *C. mediolanum* R₁₈/308 and *C. mediolanum* R₁₈/517 were inoculated with phage 39 at an m.o.i. of 1. One tube without the addition of culture was treated as control. They were then incubated on the shaker at 32°C. One ml samples were withdrawn from each tube and treated with chloroform to kill all the cells, infected or otherwise. The free phage in the adsorption mixture was assayed by the agar layer technique. The plates were incubated overnight. The number of plaques were counted and the rate of adsorption of each strain was determined.

3.23 Testing the Resistant Isolates for Lysogeny :

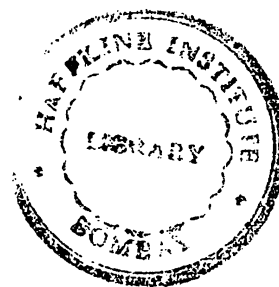
The method used to test the resistant isolates for

lysogeny was that of Clowes and Hayes (1968).

A log phase culture of the strain under study was subjected to U.V.light at a distance of 50 cms. About 7 ml of the culture in PBS was placed in a 10 cm petri dish and gently shaken at intervals. The plate was then incubated in the dark for 90-120 mins at 250°C. The supernatant was checked for free phage particles by plating out on a lawn of the sensitive host and plates incubated overnight. Presence of lysis indicates that the original culture was lysogenic.



RESULTS



R E S U L T S

I. Development of Phage Resistant Mutants :

The culture under study, C. mediolanum R₁₈ was sensitive to phage 39, a phage isolated as a aerial contaminant in the R & D laboratory of the industry. At first we attempted to develop mutants of C. mediolanum R₁₈ resistant to phage 39, using the "natural selection".

4.1 Natural Selection :

In this attempt the host C. mediolanum R₁₈ in early log phase of growth, was incubated with phage 39 at a multiplicity of infection (m.o.i.) of 10^2 - 10^4 .

Although three mutants were isolated, these were found to autolyse when grown under shaken culture conditions. When the lysate of these mutants was checked for the presence of phage, no phage could be detected.

Since the natural selection failed to yield any mutants, physical and chemical means of mutagenesis were tried out.

4.2 Physical and Chemical Mutagenesis :

4.2.1 Survival Curves :

Exposure of the organism to any mutagen, physical

or chemical, often results in exponential killing. In general, the proportions of a particular class of mutants amongst the survivors increases linearly with the dose of mutagen, till doses permitting very low levels of survivors are reached, then the proportion of mutants may fall. ‘

Thus, for each mutagen there is an optimum dose determined by a combination of mutagen concentration, time and conditions of treatment, which leaves the highest proportion of a particular class of mutants amongst survivors. This optimum dose of a mutagen that will have the highest proportion of mutant survivors can be ascertained by plotting a survival curve of the organisms against the dose of the mutagen and to select the highest dose that would result in exponential killing i.e., before any slowing down in the rate of kill with increasing dose is apparent (Hopwood, 1970).

Keeping this in view, survival curves of U.V. and NTG were plotted.

The time of exposure of C. mediolanum R₁₈ to the two mutagens was arbitrarily determined on the basis of available literature and the culture was suspended in phosphate buffered saline, pH 7.3, in place of a nutrient medium so as to avoid any variations that could arise due

to the components such as yeast a beef extract that go in the making of a nutrient medium.

Each experiment was carried out in triplicate. Table 1 and Table 2 give the mean of the number of survivors with standard error, while U.V. and NTG survival curves are represented in Figs. 1 and 2.

On the basis of these curves, the range of exposure time of 150-240 secs was selected for UV mutagenesis and a dose range of 30-100 $\mu\text{g/ml}$ was selected for NTG mutagenesis.

4.2.2 Mutagenesis :

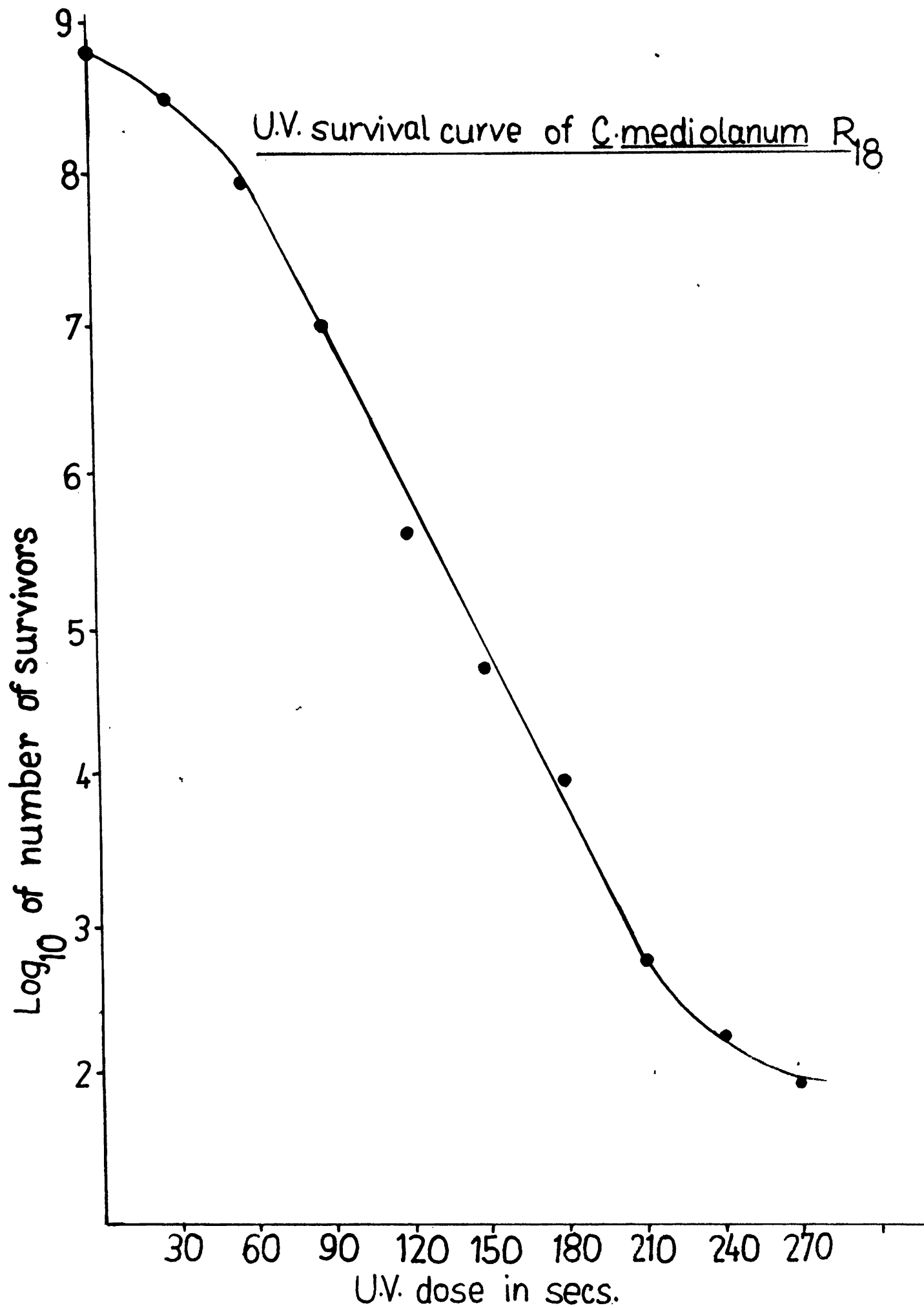
The methods used to induce mutation using U.V. radiation and NTG have been described in 'materials and methods' (Section 3.9.2 and 3.10.2). In case of C. mediolanum R₁₈, by exposing the culture to U.V. radiation for 150 to 240 secs, 41 mutants were isolated. These organisms had the desired growth rate and intact enzymatic activities. The growth rate and enzymatic activities of these mutants were tested by the R & D laboratory of the pharmaceutical industry concerned, through fermentations studies on their pilot plant and chromatographic analysis of the fermentation products respectively.

Table 1 : U.V.Survival Values of C. mediolanum R₁₈

U.V. exposure in secs.	Mean of number of Survivors/1 ml $\pm$ S.E.
0	$4.3 \times 10^8 \pm 0.54 \times 10^8$
30	$2.0 \times 10^8 \pm 0.46 \times 10^8$
60	$7.3 \times 10^7 \pm 0.54 \times 10^7$
90	$1.0 \times 10^7 \pm 0.23 \times 10^7$
120	$2.6 \times 10^5 \pm 0.69 \times 10^5$
150	$3.3 \times 10^4 \pm 0.69 \times 10^4$
180	$0.8 \times 10^4 \pm 0.12 \times 10^4$
210	$4.0 \times 10^2 \pm 0.13 \times 10^2$
240	$1.5 \times 10^2 \pm 0.23 \times 10^2$
270	$7.3 \times 10^1 \pm 0.43 \times 10^1$

Table 2 : NTG Survival Values of C. mediolanum R₁₈

NTG Dose in ug/ml	Mean of number of Survivors/1 ml $\pm$ S.E.
0	$9.0 \times 10^8 \pm 0.50 \times 10^8$
20	$2.6 \times 10^8 \pm 0.69 \times 10^8$
40	$1.0 \times 10^8 \pm 0.23 \times 10^8$
60	$6.0 \times 10^7 \pm 0.27 \times 10^7$
80	$1.6 \times 10^7 \pm 0.19 \times 10^7$
100	$1.3 \times 10^6 \pm 0.28 \times 10^6$
120	$6.3 \times 10^5 \pm 0.13 \times 10^5$



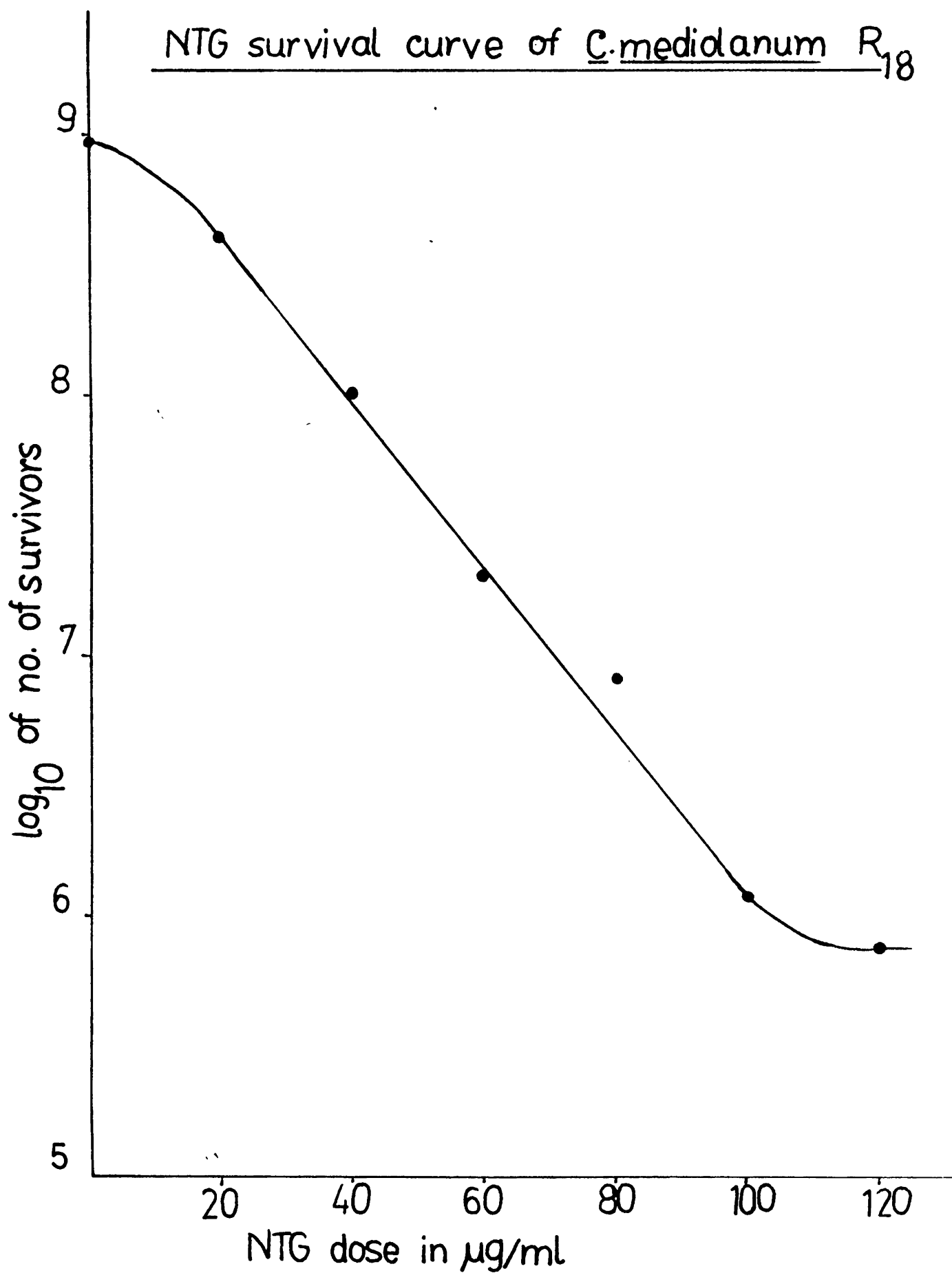


Fig.2.

Chemical mutagenesis was carried out using NTG at a dose of 30 to 100 $\mu\text{g/ml}$. This treatment yielded 26 mutants which showed no change in their growth rate and desired enzymatic activity as compared to the original sensitive strain. In addition to these, twenty seven more phage resistant mutants were isolated of which 7 converted the steroid to some other product, while the remaining twenty reverted to phage sensitivity after 3 passages on agar slants (The growth kinetics and the enzymatic activities of the mutants were checked by the R & D laboratory of the industry). The type and number of mutants isolated are summarized in Table 3.

All the above mutants were tested for lysogeny as per the method described in Section 3.23 of 'materials and methods'. None of the strains were found to be lysogenic.

II. Characterization of Phage 39 and Its Nucleic Acid :

Virus taxonomy is based on the properties of the virion and its nucleic acid.

The main criteria laid down by the International Committee on Taxonomy of Viruses are particle shape, structure, weight, bouyant density, total composition and also the nature, molecular weight and composition of viral nucleic acid (Fenner, 1976).

Table 3 : Phage Resistant Mutants of C. mediolanum R₁₈

Method employed	No. of Mutants isolated	Characteristics
Natural selection (m.o.i. 10^2 - 10^3)	3	Autolysing
U.V. Radiation (180-240 secs)	41	Resistant, showing desired enzyme activity.
	5	Resistant, converting to some other product.
NTG (40-100 μ g/ml)	26	Resistant, showing desired enzyme activity.
	7	Resistant, converting to some other product.
	20	Resistant, but reverted to sensitivity after three passages.

4.3 Electron Microscopy of Phage 39 :

The method employed in this study is described in Section 3.21.1.

The electron micrographs (Figs. 3 and 4) of the phage 39 show that the phage 39 possesses an isometric hexagonal head with a long non-contractile tail ending in a base plate. The length of the tail is more than that of the diameter of the head. There are no cross striations or fibres seen on the tail. From the morphology the phage seems to belong to the Bradley B₁ group of phages which have isometric hexagonal heads with long non-contractile tails (Bradley, 1967).

The dimensions of the phage (in mm) were measured from the micrographs, and the actual dimensions of the phage were calculated as described by Waterson (1964). (See Fig. 5 and Table 4).

4.4 Determination of the Nature of Phage Nucleic Acid :

The nature of the phage 39 nucleic acid was determined by studying its sensitivity to DNase and RNase (See Section 3.14). pBR 322 DNA and yeast RNA served as controls.

The DNase and RNase treated nucleic acids were subjected to agarose gel electrophoresis. The bands stained

Table 4 : Dimensions of Phage 39

Dimensions in Å ^o	
Phage head diameter (n = 5)	577
Phage tail (n = 5) Length x Diameter	1312.5 x 105

Where, n = The number of phage particles measured.

DIAGRAMMATIC REPRESENTATION OF PHAGE 39

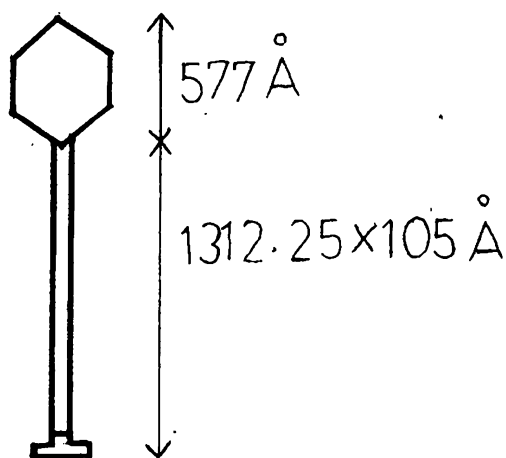
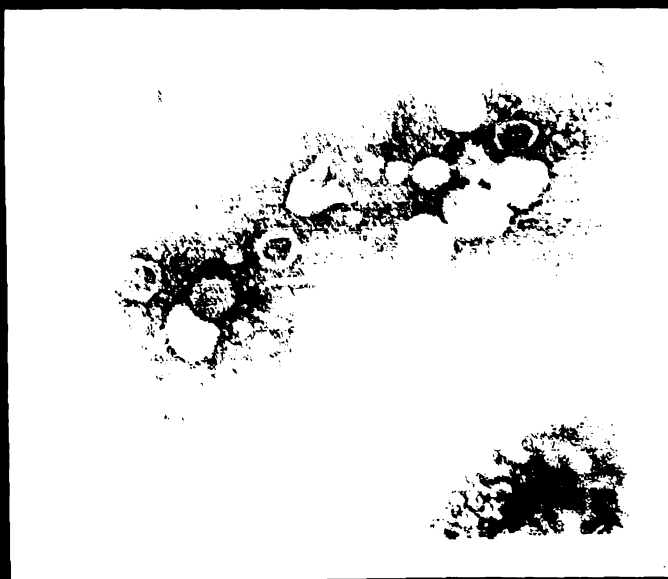


FIG. 5



Figs 3 & 4. Electron micrographs of Phage 39
(x 5,60,000, Negative staining with
uranyl oxalate).

with ethidium bromide were visualized using a U.V. source.
(See Fig. 6 and Table 5).

Table 5 : Determination of the Nature of Phage 39
Nucleic Acid

Lane No.	Sample	Result seen
1.	Phage 39 nucleic acid	Red band
2.	Phage 39 nucleic acid digested with DNase I	No band seen
3.	Phage 39 nucleic acid digested with RNase A	Red band
4.	Standard pBR 322 DNA	Red band
5.	Standard pBR 322 DNA digested with DNase I	No band seen
6.	Standard yeast RNA	Red trail seen
7.	Standard yeast RNA digested with RNase A	No trail seen

It can be seen that phage 39 nucleic acid is sensitive to the enzyme DNase I which can digest only DNA, while it does not show any sensitivity to the enzyme RNase A.

It can be inferred from this experiment that phage 39 nucleic acid is DNA.

4.5 Determination of the Structure of Phage 39 DNA :

The method used to ascertain whether the phage 39 DNA was single or double stranded consisted of agarose gel electrophoresis of the nucleic acid, followed by staining with acridine orange and visualizing the bands using a U.V. source.

Figure 7 shows the nature of phage 39 DNA when observed under U.V.light. Its details are presented in Table 6. Phage 39 DNA on staining with acridine orange showed green fluorescence under U.V. light indicating that it is double stranded.

Table 6 : Determination of the number of Strands of Phage 39 DNA.

Lane No.	Sample	Result seen
1.	Phage 39 nucleic acid	Green band
2.	ds DNA (pBR 322)	Green band

4.6 Determination of the Molecular Weight of Phage 39 DNA :

In the present study the method of co-electrophoresis on an agarose gel with standard molecular weight

markers (phage λ DNA Hind III Digest) was used for the determination of molecular weight of phage 39 DNA. The distance travelled through the gel (Fig.8) by each band was measured. The values were subjected to correction by the method of Southern (1979) to get a straight line graph. The value of m_0 was determined as given in Section 3.16 of Materials and Methods.

Table 7 summarizes the data on the mobility values of the standard and the phage 39 DNA in the gel as well as the calculated $m - m_0$ and $1/(m - m_0)$ values. From this Table it can be seen that -

$$\begin{array}{ll}
 L_1 = 15 \times 10^6 \text{ daltons} & m_1 = 1.7 \\
 L_2 = 2.83 \times 10^6 \text{ daltons} & m_2 = 3.1 \\
 L_3 = 0.37 \times 10^6 \text{ daltons} & m_3 = 6.7
 \end{array}$$

$$A = \frac{15 \times 10^6 - 2.83 \times 10^6}{2.83 \times 10^6 - 0.37 \times 10^6} \times \frac{6.7 - 3.1}{3.1 - 1.7}$$

$$A = 12.72$$

$$m_0 = \frac{6.7 - 1.7 \times 12.72}{1 - 12.72}$$

$$m_0 = 1.27$$

A graph of L --- $1/(m - m_0)$ was plotted which gives a straight line (Fig. 9). The molecular weight of phage 39 DNA as calculated from the graph is 8.5×10^6 daltons.

Determination of the MW of Phage 39 DNA

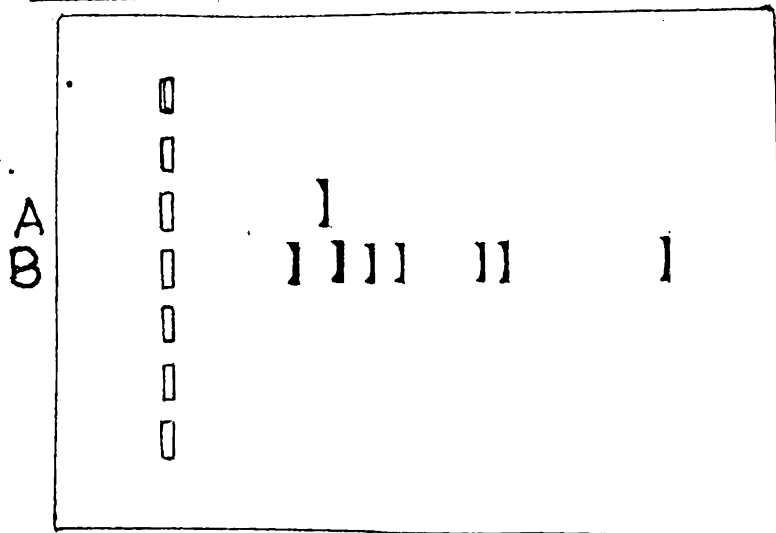


Fig. 8. Agarose gel electrophoresis of Phage 39 DNA (A), & Phage λ DNA HindIII digest (B).

Determination of the structure of Phage 39 DNA

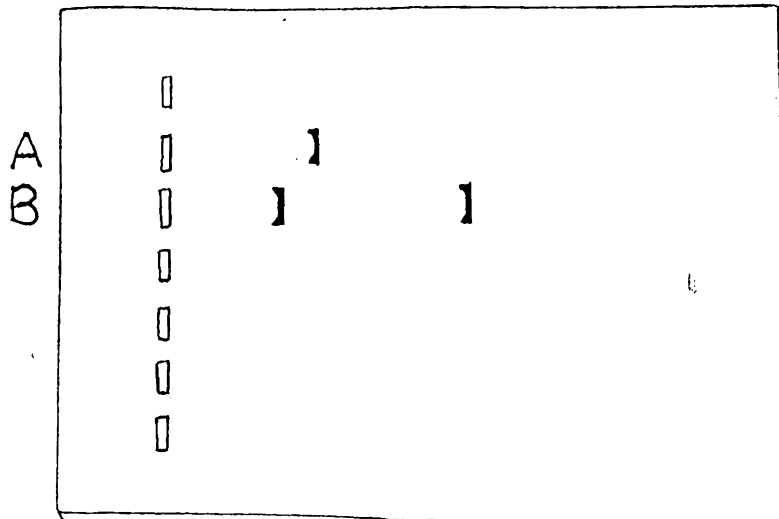


Fig. 7. Agarose gel electrophoresis of Phage 39 DNA (A), & pBR 322 DNA (B).

Table 7 : Mobilities of the Standard and Sample DNA in the Gel.

No.	Molecular Wt.* of the Sample DNA (L)	Distance Travelled by the Sample DNA (m)	$m - m_0$	$1/(m - m_0)$
<u>Standard Markers :</u>				
1.	15×10^6	1.7	0.4	2.5
2.	6.12×10^6	2.3	1.0	1.0
3.	4.26×10^6	2.7	1.4	0.71
4.	2.83×10^6	3.1	1.8	0.56
5.	1.51×10^6	4.2	2.9	0.34
6.	1.32×10^6	4.5	3.2	0.31
7.	0.37×10^6	6.7	5.4	0.19
	Phage 39 DNA	2.0	0.7	1.43

* The molecular weights of the Hind III digests of phage λ DNA are as given by Daniel et al. (1983).

Molecular weight of Phage 39 DNA

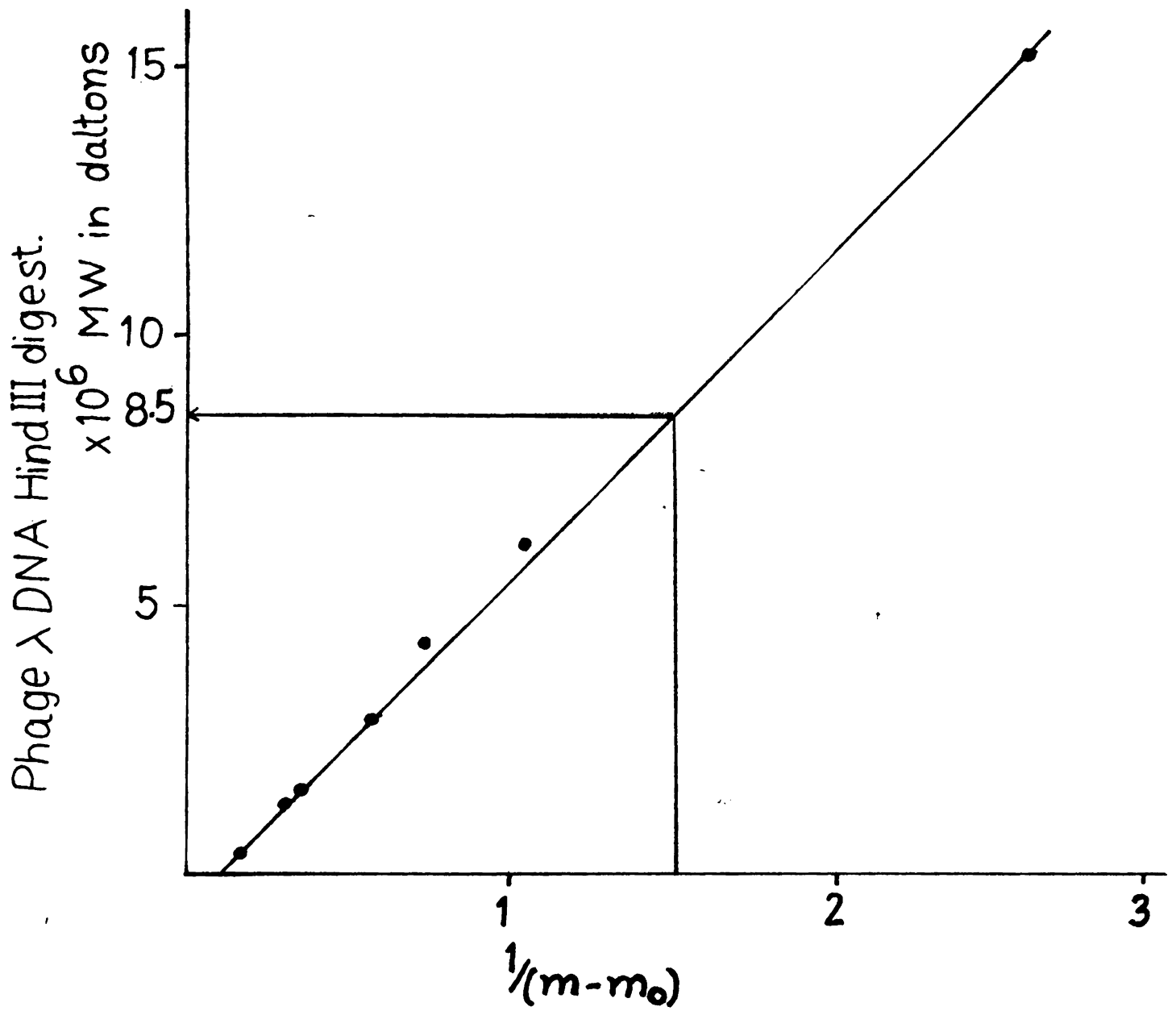


Fig.9.

Determination of the nature of Phage 39 DNA

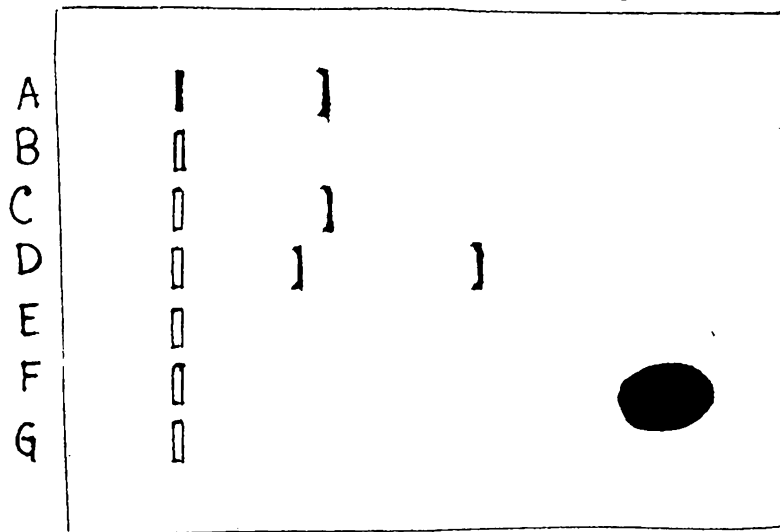


Fig.6. Agarose gelelectrophoresis of Phage 39 DNA(A), Ph 39 DNA digested with DNase (B), Phage 39 DNA digested with RNase (C), pBR 322 DNA (D), pBR 322 DNA digested with DNase (E), Yeast RNA (F) & Yeast RNA digested with RNase (G).

4.7 Determination of the Purity and Concentration of Phage Nucleic Acid :

As stated earlier (Section 2.10), the purity of a nucleic acid preparation can be ascertained by studying its absorbance spectrum over a range of wavelength 230 nm to 320 nm. Absorbance spectrum of phage 39 DNA over this range is depicted in Fig. 10 and Table 8.

The ratio of absorbance A_{260}/A_{280} was $0.197/0.117 = 1.68$ and $(A_{320}/A_{260}) \times 100$ was $(0.002/0.197) \times 100 = 1\%$. As explained in Section 2.10, these values indicate that the purity of phage 39 DNA is within the reasonable limits.

To determine the concentration of the purified phage DNA in our preparation, the DNA was suitably diluted and the concentration was determined using a standard graph (Fig. 11) wherein known concentrations of calf thymus DNA are plotted against absorbance at 260 nm. On extrapolation of the absorbance value corresponding to that of phage 39 DNA on the graph and multiplication by the dilution factor, the concentration of phage DNA was found to be $10 \times 300 = 3000 \mu\text{g/ml}$ i.e., 3 mg/ml.

Table 8 : U.V.Absorption Spectrum of Phage 39 DNA.

Wavelength in nm	Absorbance
230.	0.124
240	0.161
250	0.190
260	0.197
270	0.164
280	0.117
290	0.068
300	0.024
310	0.010
320	0.002

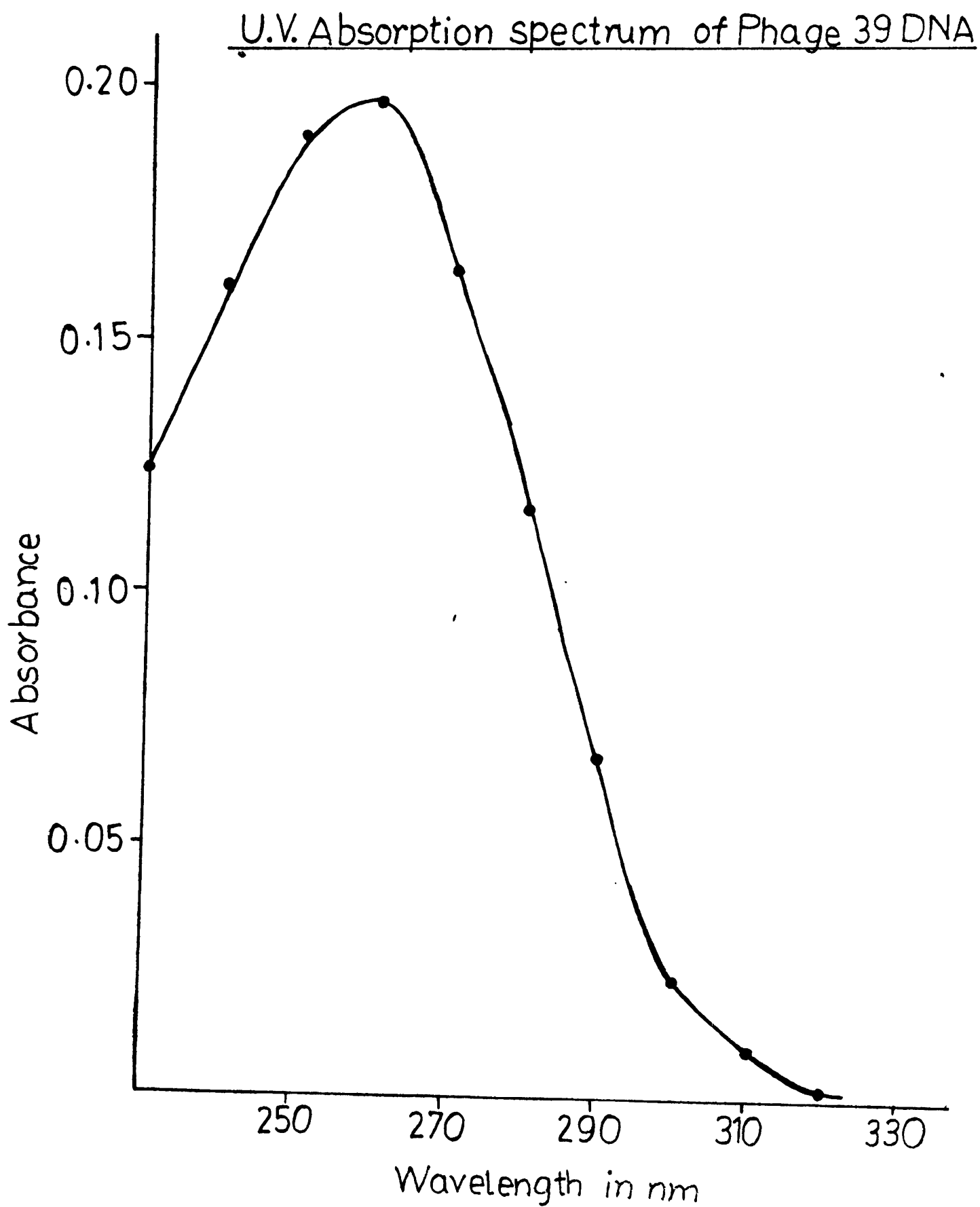


Fig.10.

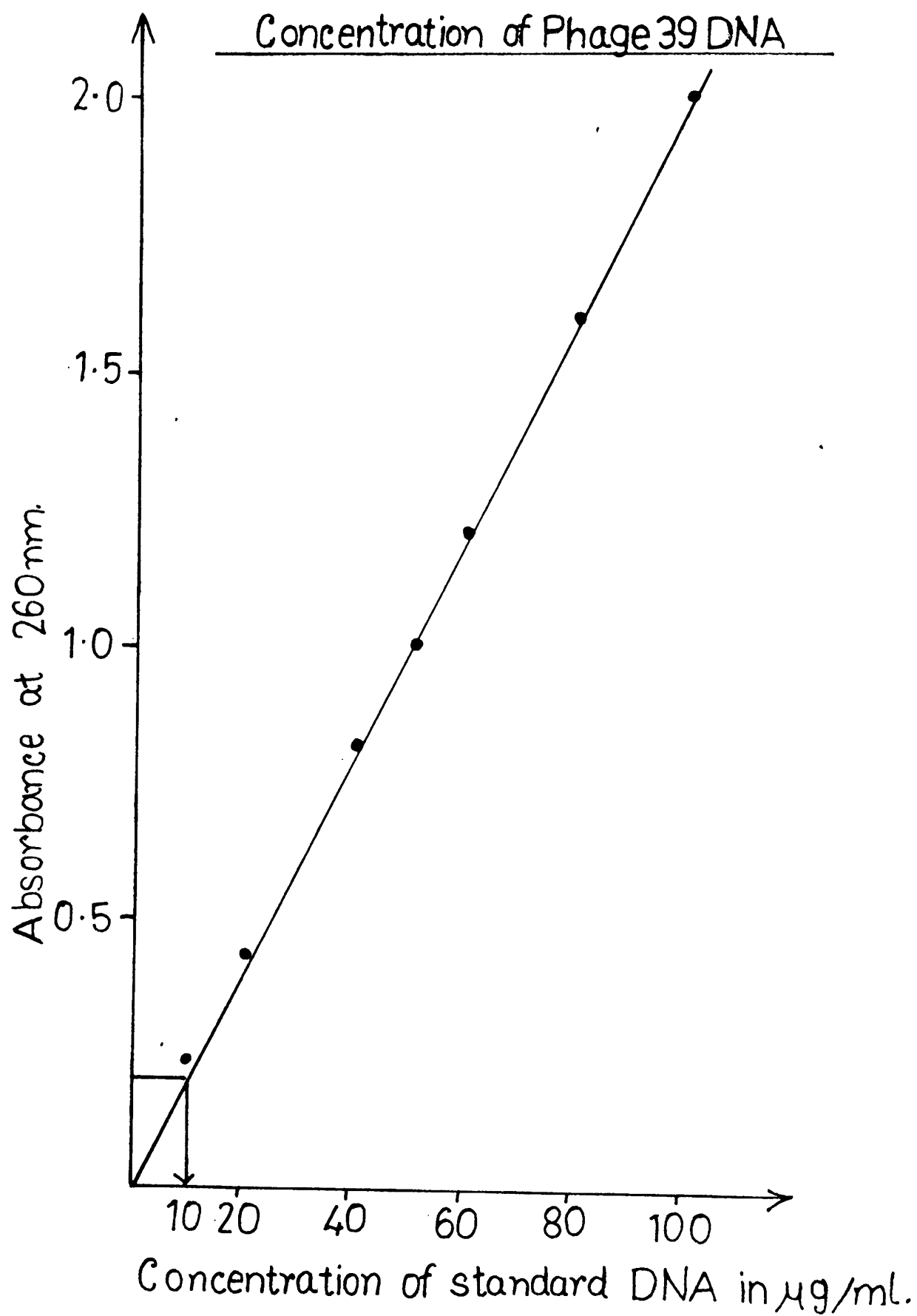


Fig.11.

4.8 One Step Growth Curve :

The overall aspects of the phage infective cycle are most conveniently studied by the one step growth experiment of Ellis and Delbruck (1939) which gives the minimum latent period of intracellular growth and average burst size of the phage.

The latent period is defined as the minimum time between the adsorption of the phage to the host cell and the lysis of the host cell with the release of phage progeny. The burst size is the mean yield of phage particles per infected bacterium (Adams, 1959).

Each of these characteristics can be measured by other techniques but the one step growth experiment enables both to be determined at the same time (See Section 3.19). The method followed here is that of Adams (1959).

It can be seen from Table 9 that plaque counts from FGT are constant through 115 min and increase suddenly by 120 mins. Therefore, the end of the latent period comes between 115 mins and 120 mins. The count from SGT increases steadily till 140 mins and remains steady thereafter. Average count during the latent period is 29. Average count at the end of bacterial lysis is 62,

Table 9.: C. mediolanum R₁₈

Plaque counts from FGT and SGT.

Time in mins.	FGT	SGT
30	40	0
60	24	1
90	27	0
95	30	0
100	23	0
105	30	0
110	27	1
115	30	1
120	550	5
125	Merged plaques	20
130	Total lysis	25
140	Total lysis	58
150		60
160		62
170		65
180		59
190		63
200		63
210		64

which when multiplied by the dilution factor of 100, gives a count of 6200 in terms of FGI. Average burst size is then $6200/29 = 213$.

Total time period from the end of the latent period to the completion of the bacterial lysis is called the rise period which is about 20 mins in the present case.

4.9 Single Burst Experiment :

The single burst experiment determines the phage yields from single cells. The phage yields from single cells may differ drastically in burst size, burst time, etc.

Here the average burst size from a single cell is calculated using a statistical approach, because there is a wide distribution in individual burst sizes (Adams, 1959).

This experiment was designed by Burnet (1929) to determine phage yield from single cells, but the method followed here is that of Adams (1959) as described in Section 3.20.

The results of the plaque assay at the end of the single burst experiment showed that 33 of the 40 plates had no plaques, while the remaining 7 plates showed a total of 1526 plaques, the individual count being 129, 141, 158, 172, 181, 208, 537.

Thirty three of the plates had no plaques hence no infected bacteria. Therefore, according to Poissons distribution theory.

$$P(0) = 43/50 = 0.825 = e^{-n} = e^{-0.19}$$

So the average number of infected bacteria per sample was 0.19 and the average number/ml of sample flask was 0.82.

If 40 samples contain an average of 0.19 infected bacteria/sample, the total number of infected bacteria in the 40 samples should have been

$$40 \times 0.19 = 7.6 \text{ Bacteria and}$$

Seven plates had plaques. This means all the 7 plates had phage yield coming from only one infected bacterium i.e., seven infected bacteria in all. These produced a total of 1526 plaques or an average burst size of $1526/7 = 218$ phage particles/bacterium.

Average plaque count/0.5 ml taken from sample flask after the end of the rise period was 100, which amounts to an average burst size of the bacteria in the sample flask of $100/0.5 = 200$.

4.10 Study of the rate of Adsorption of *C. mediolanum* R₁₈ and its Mutants :

The rate of adsorption of phage 39 on to *C. medio-*

lanum R₁₈, C. mediolanum R₁₈/308 (A mutant obtained through U.V. mutagenesis) and C. mediolanum R₁₈/517. (A mutant obtained through NTG mutagenesis) was ascertained through the assay of free phage in the adsorption mixture.

The results of the adsorption experiment in case of two representative mutant strains are summarized in Table 10. From the results it is evident that phage 39 adsorbs onto C. mediolanum R₁₈ at a rate of 99% in 5 mins, while its adsorption onto C. mediolanum R₁₈/308 and C. mediolanum R₁₈/517 is at a rate of 95% in 5 mins.

Table 10 : Adsorption of Phage 39 onto Different Hosts.

Bacterial Strain	Phage titres in PFU/ml			% Adsorption at the end of 5 mins.
	0 min	5 mins	10 mins	
<u>C. mediolanum</u> R ₁₈	1.1x10 ³	0.01x10 ³	0.001x10 ³	99
<u>C. mediolanum</u> R ₁₈ /308	1.1x10 ³	0.05x10 ³	0.01x10 ³	95
<u>C. mediolanum</u> R ₁₈ /517	1.1x10 ³	0.05x10 ³	0.01x10 ³	95

4.11 Study of Adsorption Kinetics :

A study of adsorption kinetics was not carried out in the case of C. mediolanum R₁₈ and phage 39 as the adsorption of phage 39 in 5 mins was more than 99 %.

When the proportion of phage adsorbed is more than 99% the method becomes unreliable because of incomplete sedimentation of infected bacteria during centrifugation period, which may lead apparently to false high values for free phage. Besides, the assay of free phage done by agar layer technique is not so sensitive in order to register differences in adsorption rate beyond 99 % (Adams, 1959).



DISCUSSION

D I S C U S S I O N



During the course of this work mutants of C. mediolanum R_{18} resistant to phage 39 were developed. The study also included characterization of phage 39 and study of the phage resistant mutants isolated.

The dose of each mutagen i.e., U.V.radiation and NTG was selected after plotting survival curves of C. mediolanum R_{18} . Exposure of the organism to any mutagen often results in exponential killing. Killing in haploid organisms is exponential, marked by an initial lag probably atleast in part due to the operation of repair mechanisms. For any mutagen the number of viable mutants in a population will increase with the dose of the mutagen upto a certain level and then fall as the rate of killing overtakes the rate of induction of new mutants. In other words, the proportion of mutants amongst survivors increases with the dose of the mutagen and when a critical dose is reached, the proportion of mutants often begins to fall. In some instances this is due to the genetical or physiological heterogeneity of the original population in respect of sensitivity to killing and mutagenic effects of the mutagen, so that at high doses the more resistant components come to make up an increasing proportion of

survivors (Hopwood, 1970).

On plotting a U.V. survival curve of C. mediolanum it was found that the exposure to U.V. for a period above 180 secs which caused more than 99.99% kill yielded phage resistant mutants.

On plotting the NTG survival curve of C. mediolanum R_{18} , it was found that the dose (40 ug/ml and above) which resulted in more than 88.89% kill yielded phage resistant mutants.

Of the 53 mutants isolated using NTG, twenty mutants reverted to phage sensitivity after a few passages on agar slants. This is probably explained by the fact that the phage sensitivity of a strain is a function of two separately evolving genetic entities; the phage and the bacterium. Any change in the population of phage due to host controlled modification, changes in the genetic make up of bacteria due to mutations or by loss of unstable phage-defence mechanisms can lead to an overall change in the phage resistance pattern of the strain (Sanders and Klaenhammer, 1983).

As mentioned earlier in Section 2.2, resistance to phage infection could be due to interference in the adsorption of the phage onto the host cell surface,

penetration of the phage nucleic acid or the intracellular transcription/replication of phage nucleic acid.

The resistance to phage attack in case of C. mediolanum R₁₈/308 and C. mediolanum R₁₈/517 seems to result from either the inability of the phage 39 DNA to penetrate the cell or interference at the intracellular level. The resistance in this case is not due to the loss or alteration of the cell wall receptors, as the phage adsorbs the mutants to more or less the same extent as the sensitive strain i.e., 95% adsorption in 5 mins in case of the mutants and 99% adsorption in 5 mins in case of the sensitive strain.

Very little is known about the aberrations at the penetration level. It has been observed by Labedan (1978) that variants of phage T5 are blocked in the entry of their DNA into the host through modification of the cell membrane of the host. Phage resistance may also be due to the action of specific nucleases that restrict the DNA of the phage. Such was the case observed by Hattman and Fukasawa (1963) in case of T-even coliphages. The DNA of the T-even coliphages is broken down by the specific nucleases of the mutant host E. coli which is unable to synthesise uridine diphosphoglucose, the glucosyl donor for phage DNA.

Once inside the host cell the onset of the lytic cycle of the phage DNA can be blocked by a host mutation which has resulted in the production of a mutant RNA polymerase. Snyder and Montgomery (1974) have reported inhibition of T4 growth by an RNA polymerase mutation of E. coli due to failure of RNA polymerase to perform a reaction(s) which physically alters or changes the association of parental and probably some progeny DNA. This reaction is required early for the T4 DNA replication and throughout infection for late gene expression.

The lytic activity can also be blocked through modifications of certain host enzymes involved in the processing or assembling of phage proteins (Coppo et al., 1974). Thus, the resistance in case of C. mediolanum R₁₈ could result from the interference with entry of or effective replication/transcription of phage DNA or the synthesis assembly of phage coat proteins.

The electron microscopy of phage revealed that the phage belongs to the Bradley B1 group of phages. Phage T5 is also a member of this group. The T5 head measures 650 Å in diameter and the tail measurements are 1700 x 100 Å (McCorquodale, 1975). These are comparable to the values for phage 39, the head diameter being 577 Å and the tail measuring 1312.25 x 105 Å .

S U M M A R Y

The thesis deals with development of phage-resistant variants of an industrially important culture which is used for "steroid transformation". Some of the phage characteristics as well as the mechanism involved in the development of "phage resistance" was studied.

1) Phage resistant mutants of C. *mediolanum* R₁₈ were developed using U.V. radiation and NTG.

2) Electron micrographs showed that phage 39 belonged to Bradley B1 group having the following dimensions : $577 \overset{\circ}{\text{Å}} \times 1312.25 \overset{\circ}{\text{Å}} \times 105 \overset{\circ}{\text{Å}}$. The phage 39 was found to contain double stranded DNA as its genetic material through observations based on enzyme sensitivity and acridine orange staining of the purified nucleic acid. The molecular weight of phage 39 DNA was determined to be 8.5×10^6 daltons through co-electrophoresis of the phage DNA on an agarose gel with standard molecular weight markers. The latent period of phage 39 infection was found to be 20 mins and the average burst size 213.

3) The mutants seem to resist phage either by blocking the penetration of phage DNA or by interfering

with the replication/transcription of the phage DNA if the phage DNA penetration is unaffected. Adsorption studies have revealed that the phage adsorbs normally to the mutants indicating that the phage receptors on the cell surface of the mutants are neither lost nor altered.

A P P E N D I X

Media and Reagents :

It may be noted that all media, buffers and solutions unless otherwise mentioned were sterilized by autoclaving at 121°C for 15 mins and stored at room temperature.

1. F₁₈ Broth :

Yeast extract (Oxoid)		10.0	g.
Na ₂ HPO ₄ ·2H ₂ O		4.5	g.
K ₂ HPO ₄		3.4	g.
d/w		1000	ml.
pH		6.6	

2. F₁₅ Agar :

F ₁₈ broth		100.0	ml.
Agar		2.0	g.

3. F₁₅ Soft Agar :

F ₁₈ Broth		100.0	ml.
Agar		0.8	g.

4. Phosphate buffered saline :

K ₂ HPO ₄		1.21	g.
KH ₂ PO ₄		0.34	g.
NaCl		8.0	g.
d/w		1000	ml.
pH		7.3	

5. NTG Stock Solution : 1.0 mg/ml.

NTG (Sigma)		10.0	mg.
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d/w (Sterile)		10.0	ml.
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Stored at 4°C.

6. DNase solution : 100.0 µg/ml.

DNase I (Cal Biochem from bovine pancreas, b grade)		1.0	mg.
--	-------	-----	-----

0.02M Sodium acetate (sterile) (pH 5.2)		10.0	ml
--	-------	------	----

Dispensed in aliquots and stored
at -20°C.

7. RNase A Solution : 100 µg/ml.

RNase A (Sigma, from bovine pancreas, type X1IA)		1.0	mg
---	-------	-----	----

20 mM Tris-HCl (pH 7.5) (Sterile) ..		5.0	ml
--------------------------------------	--	-----	----

30 mM NaCl (Sterile)		5.0	ml
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Dispensed in aliquots and stored
at -20°C.

8. SM Buffer :

NaCl		5.8	g.
------	-------	-----	----

MgSO ₄ ·7H ₂ O		2.0	g.
--------------------------------------	-------	-----	----

1M Tris-HCl (pH 7.5)		50.0	ml.
----------------------	-------	------	-----

2% Gelatin		5.0	ml.
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Dispensed in aliquots and sterilized.

9. Proteinase K : 50 μ g/10 μ l.

Proteinase K (Sigma from
Tritirachium album Type XI) 5.0 mg.

d/w (Sterile) 1.0 ml.

Dispensed in aliquots and stored
at -20°C .

10. 20% Sodium dodecyl sulphate (SDS) :

SDS 2.0 g.

d/w 10.0 ml.

This solution did not require sterilization for
its storage at room temperature.

11. 0.5 M EDTA (pH 8.0)

EDTA 18.6 ml.

d/w 80.0 ml.

The pH was adjusted to 8 by NaOH pellets and the
volume was made upto 100 ml. The solution was dispensed
in aliquots and sterilized.

12. TE buffer (pH 8.0) :

1 M Tris-HCl (pH 8.0) 10.0 ml.

0.5 M EDTA (pH 8.0) 2.0 ml.

d/w 988.0 ml.

The buffer was dispensed in aliquots and
sterilized.

13. Siliconization :

The Eppendorf tubes, pipette tips and glassware were

siliconized with Sigma silicone cote. The washed and dried glasswares and plastic wares were coated with silicone cote and allowed to dry for 1-2 hrs. These were then immersed in d/w to remove excess of cote and sterilized by autoclaving at 121°C for 15 minutes, before using.

14. Phenol :

Phenol was distilled and stored at -20°C . Whenever required 55 ml of 1M Tris (pH 8.0) was added to 500 g of phenol crystals and the bottle was kept in warm water to melt the crystals. On melting a further 110 ml of 1M Tris (pH 8.0) was added. On cooling 8-hydroxy quinoline (0.1%) was added to prevent oxidation of phenol and inhibit impurities. The melted phenol was then extracted several times with an equal volume of buffer (1M Tris HCl, pH 8.0), followed by 0.1M Tris (pH 8.0 and 0.2% mercapto-ethanol) until the pH of the aqueous phase was more than 7.6. The phenol was then stored at 4°C under equilibration buffer (TE buffer, pH 8.0).

15. Chloroform :

Chloroform was double distilled and a mixture of chloroform : isoamyl alcohol (24:1) was prepared for use in the extraction of phage nucleic acid. The mixture was stored at room temperature.

16. Isopropanol :

It was double distilled and stored at room temperature.

17. Ethanol :

Absolute alcohol was double distilled and stored at -20°C .

18. 0.8% Agarose :

Agarose (Fluka)		0.24	g.
50X TAE buffer		0.6	ml.
d/w		29.4	ml.

19. 50X TAE buffer :

Tris-HCl		24.2	g.
Glacial acetic acid		5.7	ml.
0.5M EDTA (pH 8.0)		10.0	ml.
d/w		84.3	ml.

Stored at room temperature.

20. 6X Gel loading buffer :

Bromophenol blue		10.0	mg.
Sucrose		2.0	g.
d/w		5.0	ml.

Stored at 4°C .

21. Ethidium bromide : 100 $\mu\text{g/ml}$.

EtBr (Sigma)		1.0	mg.
d/w		10.0	ml.

Stored at 4°C in a foil wrapped bottle.

22. 1 mM MgSO₄ · 7H₂O :

MgSO ₄ · 7H ₂ O		0.14	mg.
d/w		100	ml.

23. 10 mM Sodium phosphate buffer :

NaH ₂ PO ₄		1.2	g.
Na ₂ HPO ₄		1.4	g.
d/w		1000	ml.
pH		7.0	

24. Acridine orange :

Acridine orange (BDH)		3.0	mg.
10 mM Sodium phosphate buffer		100	ml.

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SYNOPSIS

INTRODUCTION

Microbial transformation of organic compounds has been known to man in an empirical way since the dawn of history. Microorganisms have been employed in the production of beer, wine, pickling of certain plant materials, leavening of bread, making of vinegar, cheese, butter, retting of flax, etc..

With the advent of microbiology, several new industries have emerged wherein microorganisms are used for the synthesis of vital metabolites, such as amino-acids, vitamins, antibiotics, enzymes and in the bio-conversion of specific organic molecules, such as the transformation of steroids. Microorganisms owe this ability for synthesis and bioconversions to the enzymes present in their make-up.

Efficient transformation of steroids demands optimal physical and chemical conditions (i.e., pH, aeration, agitation and rich nutrient medium) for the growth of microorganisms in the fermentation tank. With the culture in the actively growing phase, the steroid is 'charged' into the tank for conversion. Adequate time is allowed for the conversions to take place, the physical and chemical conditions in the fermentor as well as the cell mass being carefully monitored. Finally, the product is harvested and purified.

However, these attempts can be foiled by a bacterial virus, which can attack the growing bacterial culture, resulting in a sharp decline in the conversion rate. The bacterial viruses or bacteriophages (phages in short) are extremely small in size ($< 0.2 \times 0.07 \mu$) and can be carried across the filters used to sterilize the air introduced into the fermentor, although these filters can effectively block the passage of intact bacterial cells. Phages thus pose a great threat to such fermentation processes, as their ability to be carried by dust in the air, allows them to infiltrate into the fermentor.

Once the phage reaches its host, it destroys its host through the following steps :-

- 1) Adsorption of the phage to a specific receptor on the host.
- 2) Penetration of the phage nucleic acid into the host.
- 3) Multiplication of the phage in the host.
- 4) Lysis of the host, releasing mature phage particles.

Since a phage attack is an ever present possibility, ways and means to combat this interference in fermentations have been developed. One can achieve this

by making use of phage resistant mutants or by utilizing antiphage agents in the fermentation medium. A variety of methods are now available to raise mutant bacteria, viz., bacteria with a change in the reading frame of their DNA, thereby causing a phenotypic change expressed through proteins.

Interference in any one of the steps involved in phage infection listed above, can lead to phage resistance. While use of an antiphage agent, in the fermentation medium effectively blocks the adsorption of the phage on to the host bacterial cell, mutation to phage resistance may involve interference with adsorption and penetration of the phage nucleic acid into the host cell or replication of the phage nucleic acid inside the host cell.

Mutations can occur spontaneously during phage reproduction at a low frequency (10^{-6} to 10^{-8}). The frequency can be increased by physical and chemical mutagenesis. This involves the use of agents such as ionizing radiations (X rays, γ rays and neutrons), non-ionizing radiations (U.V. radiations), base analogues (5-Bromo-uracil, 2-amino-purine), alkylating agents (Ethyl-methane sulphonate, Methyl-methane sulphonate, N-methyl N'-nitro N-nitrosoguanidine).

A pharmaceutical concern in Bombay carrying out bioconversion of steroids, uses several bacterial and fungal cultures to affect modifications at different sites on the steroid molecule. Some of these cultures are susceptible to their phages and therefore, the unit had been experiencing phage attacks on their plant, thus incurring heavy losses. One of the cultures being used for steroid transformation was Corynebacterium mediolanum. It was of interest to develop phage resistant mutants that could help the industry and of even more interest was studying the phage and host and the mechanism involved in the development of resistant cultures and comparing it with the information on other phage resistant strains developed in our laboratory in an earlier study.

The following were the precise aims and objectives of this study :-

- 1) To develop phage resistant mutants which have retained their desired enzymatic activity.
- 2) To study the mechanisms involved in the resistance of these mutants.
- 3) To purify and characterise the phage and its nucleic acid.

MATERIALS AND METHODS

The organism and the phage

The organism used during the course of the study was Corynebacterium mediolanum R₁₈.

The phage used during the course of this study was phage 39.

Maintenance and Preservation of Cultures and Phage 39

The media for the organisms used during the course of this study were nutrient broth/agar, or one based on yeast extract, sodium phosphate dibasic and potassium dihydrogen phosphate. The bacterial strains were preserved in a lyophilised state. The organism was grown in broth (50 ml in a 250 ml Erlenmeyer flask) on a shaker at 32°C.

The phage 39 was maintained in broth at 4°C. Chloroform was added to the phage lysate to prevent bacterial contamination. The phage was also maintained on agar plates by making occasional transfers from a master plate of the phage to a lawn of the sensitive host. Phage titres were determined by the universally accepted agar layer method (Adams, 1959).

Development of Phage Resistant Mutants

The methods used for the development of phage resistant mutants were :

- 1) Natural selection
- 2) Physical mutagenesis
- 3) Chemical mutagenesis.

Standard procedures described by Clowes and Hayes (1968) and Hopwood (1970) were used in case of each treatment.

Natural selection

Natural selection was carried out by exposing a log-phase culture of C. mediolanum R₁₈ to high titre phage suspension. The sensitive cells were killed, leaving behind the naturally resistant cells, which were then plated out on agar plates. The colonies that appeared were tested for phage resistance.

Physical and chemical mutagenesis

The biological effect of the mutagen^e can be usually inferred from the slope and shape of the curves constructed by plotting the number of surviving organisms as a function of the dosage (Hayes, 1968). The proportion of a particular class of mutants amongst survivors, generally increases approximately linearly with the dose,

until at doses giving very low levels of survival the proportion may fall. A guide to determining the dose of mutagen that will have the highest proportion of mutant survivors, is to plot a survival curve and to use the highest dose that results in exponential killing i.e., before any slowing down in the rate of kill with increasing dose is apparent (Hopwood, 1970).

Among the known physical mutagens as mentioned earlier, U.V. radiation was the mutagen selected to develop phage resistant mutants. It is extremely convenient mutagen as its mutagenic wavelength lies between 200-300 nm (the peak of absorption by nucleic acids lies well within this range).

Innumerable compounds are mutagenic but very few are convenient for routine isolation of mutants. In this study N-methyl N'-nitro N'-nitrosoguanidine (NTG) was used for developing phage resistant mutants. NTG is one of the most drastic mutagens known. Under optimal conditions, it gives high mutant yield with comparatively little killing.

Study of the Mechanism of Resistance

As mentioned in the introduction, resistance to phage could develop through interference with any one of the steps involved in the lytic cycle.

Detection of the free or unadsorbed phage in the adsorption mixture is one of the means to determine the mechanism of resistance involved in the mutants. The free or unadsorbed phage in the adsorption mixture was determined by the method of Adams (1959).

Purification of the Phage

The phage 39 was purified by the method of Maniatis et al. (1982a).

Extraction of Phage Nucleic Acid

The phage nucleic acid was extracted and purified by the method of Maniatis et al. (1982b).

The concentration and purity of the nucleic acid was determined through the absorption spectrum of the nucleic acid and extrapolation on the graph of standard DNA.

Characterisation of Nucleic Acid

a) Type of nucleic acid : The nature of the nucleic acid was determined using the method of McMasters and Carmichael (1977) and Aaij and Borst (1972).

b) Molecular weight of the phage nucleic acid : The extent of migration of a DNA molecule in an agarose gel is

a function of its molecular weight over a broad range. The length of an unknown fragment can be closely determined by coelectrophoresis with other fragments of known length (Schleif, 1981).

The molecular weight of the phage nucleic acid was determined by running it on an agarose gel with standard molecular weight markers of λ Hind III DNA digest using the method of Southern (1979).

Electron Microscopy

The electron micrographs of phage 39 were taken with the kind help of Dr. A.N. Bhisey, Head, Department of Cell Biology, Cancer Research Institute.

Negative staining with 0.5% uranyl oxalate on formvar and cytochrome c coated grids was used to prepare the specimen for electron microscopy (Tikhonenko, 1970).

SUMMARY OF RESULTS

Development of Phage Resistant Mutants

Three mutants of C. mediolanum R₁₈ were isolated through natural selection, but they showed a tendency to autolyse, hence, they were discarded.

Before proceeding to induced mutagenesis survival curves of the culture against both U.V. and NTG were plotted. Based on these curves, exposure time of 150 to 240 secs was selected for U.V. and the dose range of 30 to 100 $\mu\text{g/ml}$ was selected for NTG.

41 Stable enzymatically active phage resistant mutants were isolated using U.V. and another 26 using NTG. In addition, U.V. treatment yielded 5 mutants which lacked the desired enzymatic activity; while, NTG treatment yielded 7 such mutants (The enzymatic activity was checked by the R and D laboratory of the Pharmaceutical concern). NTG treatment yielded 20 mutants that reverted to phage sensitivity after few passages and hence, these were discarded.

Study of the Mechanism of Resistance

It was found that phage 39 adsorbed on to C. mediolanum R_{18} mutants normally. 95% of the phage particles adsorbed on to the resistant mutants within 5 mts as against the 99% within 5 mts on to the sensitive R_{18} .

The mechanism involved in the resistance of these mutants, therefore, involves either the inability of the phage nucleic acid to penetrate into the host, or the

inability of the phage nucleic acid to replicate in the host.

Purification of Phage 39

The purification procedure raised the phage titre (measured as plaque forming units, PFU) from 3×10^{10} PFU/ml in the original lysate to 6×10^{12} PFU/ml.

Phage Nucleic Acid

The nucleic acid concentration of phage 39 determined through extrapolation on a graph of standard DNA was 3 mg/ml.

Through agarose gel electrophoresis and staining with acridine orange and ethidium bromide, the nature of the phage nucleic acid was found to be of dsDNA.

The molecular weight (by the method of Southern (1979) was 8.5×10^6 daltons.

Electron Microscopy of Phage 39

Electron microscopy of the phage revealed that the phage 39 possesses a polyhedral head and a long flexible tail with spikes. The phage thus belongs to the Bradley B₁ group of phages (Bradley, 1967).

The dimensions of the phage 39 as determined from the electron micrographs were as follows:.....

Phage head diameter	- 577 A ⁰
Tail length	- 1312.25 A ⁰
Tail diameter	- 105 A ⁰

The above results, as well as the relationship between the different cultures employed in the steroid transformations and their phages will be discussed in the thesis.

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