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ISOLATION AND PARTIAL CHARACTERIZATION OF BACTERIOPHAGES

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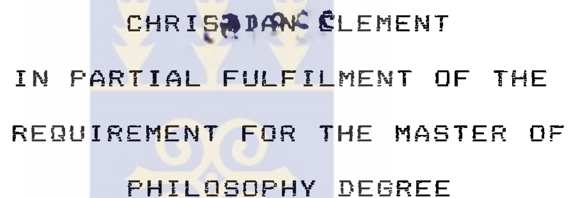

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IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE MASTER OF PHILOSOPHY DEGREE

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
UNIVERSITY OF GHANA
LEGON.

SEPTEMBER 1993.

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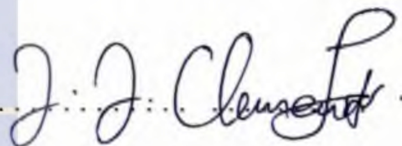
DECLARATION

THE WORK DESCRIBED IN THE REPORT WAS CARRIED OUT BY ME AT THE DEPARTMENT OF BIOCHEMISTRY UNIVERSITY OF GHANA, LEGON, UNDER THE SUPERVISION OF DR. FREDERICK N. GYANG AND DR. FREDERICK K. RODRIGUES.

DATE 24TH NOV., 1993 SIGNATURE 

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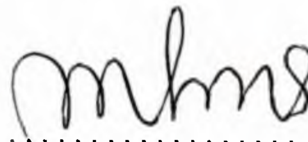
DATE Feb 10, 1995 SIGNATURE 

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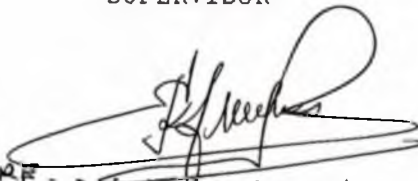
DATE 24TH NOV., 1993 SIGNATURE 

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DEDICATION

TO MY MOTHER MRS. JULIANA AKUOKOR CLEMENT IN APPRECIATION FOR HER LOYAL AND UNFLINCHING SUPPORT TO ME FROM MY INFANCY TO PRESENT.

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ACKNOWLEDGEMENTS

It is with unqualified gratitude and a deep sense of respect that I acknowledge here the invaluable support of all those who contributed to the successful completion of this work.

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To my colleagues, Felix Charles Mills-Robertson, Alex Owusu-Biney and Nii Ayite-Aryee, I can only say a BIG thank you.

Finally to Mrs. Christiana Nettey who typed my work, I say thank you from my heart and that her contribution to this work is inscribed in 'gold'.

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GLOSSARY

Icosahedral	-Polyhedron with 20 triangular faces and 12 vertices.
Isometric	-Cube or regular octahedron characterised by three equal axes at right angles.
DNA	-Deoxyribonucleic Acid.
p.f.u.	-Plaque-forming Units.
Capsid	-Protein Shell surrounding the centrally located nucleic acid of the virus.
SM	-Medium for storage and dilution of bacteriophages.
SDS-PAGE	-Sodium Dodecylsulphate Polyacrylamide Electrophoresis.
TE	-Tris Ethylenediamine Tetra-acetic Acid.
MIC	-Minimum inhibitory concentration.

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TABLE OF CONTENTS

Page

DECLARATION i

DEDICATION ii

ACKNOWLEDGEMENTS iii

GLOSSARY iv

TABLE OF CONTENTS v

ABSTRACT viii

CHAPTER ONE - INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION AND LITERATURE REVIEW 1

1.1. 1 GENERAL INTRODUCTION 1

1.1. 2 PHAGE MORPHOLOGY 3

1.1. 3 PHAGE NUCLEIC ACIDS 6

1.1. 4 PHAGE PROTEIN COAT 9

1.1. 5 THE PHYSIOLOGY OF PHAGE INFECTION 12

1.1. 6 ATTACHMENT AND PENETRATION 12

1.1. 7 HOST CELL FUNCTION AND PHAGE GENE EXPRESSION . 13

1.1. 8 PHAGE ASSEMBLY AND DNA PACKAGING 14

1.1. 9 PHAGE DNA: MOLECULAR STRUCTURE AND BASE COMPOSITION 16

1.1. 10 RESTRICTION AND MODIFICATION OF DNA 18

1.1. 11 TEMPERATE AND VIRULENT BACTERIOPHAGES, LYSOGENY AND TRANSDUCTION 23

1.1. 12 BACTERIAL-PHAGE RELATIONSHIP 27

1.1. 13 OBJECTIVES 28

CHAPTER TWO - MATERIAL AND METHODS

2.1 MATERIALS AND METHODS 31

2.1. 1 MATERIALS 31

University of Ghana http://ugspace.ug.edu.gh

2.1. 2 SOURCES OF BACTERIAL STRAINS 32

2.2 METHODS 33

2.2.1 MAINTENANCE OF BACTERIAL CULTURES. . . . 33

2.2. 2 ISOLATION OF BACTERIOPHAGES 33

2.2. 3 PROPAGATION OF BACTERIOPHAGES 34

2.2. 4 TITRATION OF BACTERIOPHAGES 35

2.2.5 PURIFICATION OF BACTERIOPHAGES 35

2.2. 6 CHARACTERIZATION OF BACTERIOPHAGES 36

2.2. 7 ELECTRON MICROSCOPY OF BACTERIOPHAGES 36

2.2. 8 BACTERIOPHAGE ANTISERA PREPARATION 37

2.2. 9 BACTERIOPHAGE INACTIVATION 38

2.2. 10 ISOLATION OF DNA FROM BACTERIOPHAGES 39

2.2. 11 RESTRICTION OF BACTERIOPHAGE DNA 40

2.2. 12 AGAROSE GEL ELECTROPHORESIS OF BACTERIOPHAGE DNA 40

2.2. 13 SOLUBILIZATION OF BACTERIOPHAGE PROTEINS . . . 41

2.2. 14 SDS-PAGE OF BACTERIOPHAGE PROTEINS . . . 41

2.2. 15 DETERMINATION OF MINIMUM ANTIBIOTIC INHIBITORY CONCENTRATIONS (MIC) OF THE BACTERIAL STRAINS . 42

2.2. 16 INDUCTION OF INCREASED ANTIBIOTIC TOLERANCE IN BACTERIAL STRAINS 43

2.2. 17 DETERMINATION OF TRANSDUCING ABILITY OF BACTERIOPHAGES 44

CHAPTER THREE - RESULTS

3.1 RESULTS 46

3.1. 1 ISOLATION OF BACTERIOPHAGES 46

3.1. 2 SEROLOGICAL PREPARATION 65

3.1. 3 DIGESTION OF PHAGE DNA WITH RESTRICTION ENDONUCLEASE HIND III AND ECO RI 68

3.1. 4 SDS-PAGE OF SOLUBILIZED PHAGE PROTEINS . . 75

University of Ghana http://ugspace.ug.edu.gh

3.1. 5 MINIMUM ANTIBIOTIC INHIBITORY CONCENTRATION (MIC) AND INDUCTION OF INCREASED ANTIBIOTIC TOLERANCE IN BACTERIAL STRAINS 79

3.1. 6 TRANSDUCTION 88

CHAPTER 4 - DISCUSSION AND CONCLUSION

4.1. DISCUSSION AND CONCLUSION 91

4.1. 1 DISCUSSION 91

4.1. 2 CONCLUSION 99

BIBLIOGRAPHY 101

University of Ghana http://ugspace.ug.edu.gh

ABSTRACT

Bacteriophages were isolated from sewage samples obtained from the Tema Sewage Disposal Plant and the Legon vicinity using the indicator bacteria strains Shigella dysenteriae, Salmonella typhi, Escherichia coli, and Salmonella group D. Ten phages were isolated and partially characterized by electron microscopy, DNA fingerprinting and protein profiles by SDS-PAGE.

The relatedness of the phages was determined by immunological studies. Some of the phages were screened for their ability to transduce antibiotic resistance markers.

The phages were found to be similar to T-even phages and P phages. The morphology possessed by a phage was independent of the host specificity, that is phages of different morphologies could attack one bacteria strain.

The restriction fragment lengths of the phage DNA ranged between 0.125-33.113 kilobasepairs (kbp) and one fragment of fragment length 23.000kbp was common to nine phages, suggesting a relationship between phage nucleic acid and morphologies.

Phage EcRCL24 for which antisera was raised was found to show an appreciable cross reactivity with the other EcRCL phages and they are therefore closely related.

The phages are presumed to be capable of generalized transduction and the frequency of 3 x 10^{-6} for penicillin-V-sulfoxide and 3 x 10^{-7} for tetracycline and erythromycin

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suggests a relatedness of the indicator bacteria strains' chromosomes.

The additional information obtained within the scope of the project, involved comparison of minimum inhibitory concentration (MIC) values and those obtained by the induction of increased tolerance levels of antibiotics in the clinical isolates. It showed that, these pathogens could tolerate about double their MIC values in the increased tolerance level experiments. Furthermore their MIC values were considerably higher than the chemotherapeutic dosages.

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CHAPTER 1

1.1 INTRODUCTION AND LITERATURE REVIEW

1.1.1 GENERAL INTRODUCTION

Viruses are macromolecular particles whose genomes are either DNA or RNA. They are biologically passive but multiply after they have entered a host-specific cell. Viruses use their synthesizing machinery to direct the production of specialized particles, the virions inside the living cells.

There are three major types of viruses based on their host specificity or the organisms they attack namely plant, bacterium and animal viruses. Bacteriophages or bacteria viruses were originally isolated in 1915 by Twort, a British scientist (Salle, 1961) and further studied by d'Herelle, a French investigator who coined the word "bacteriophage" which literally means "bacterium eater" (Smith, 1963).

Bacteriophages can be isolated from the natural environment, such as soil, water, sewage, dung, rotten vegetation, sheep and cattle droppings. The classification of phages is based on morphology, chemical structure, type of nucleic acid, and their interaction with the host bacterial cell. The relation of phages to sex differentiation of bacteria is taken into account in determination of additional taxonomic signs. It has been established that one group of phages affects only male bacteria (F^+), another group only female bacteria (F^-), while a third group of phages is indifferent with respect to sex differentiation of cells (Pyatkin and Krivoshein, 1987). Phages are marked by a

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specific effect on the corresponding bacterial species. Each phage has its own host in which it lives and multiplies. Staphylococci have 40 phage types, *Escherichia coli* 50, *Salmonella typhi* 56, *S. paratyphi A* 11, *S. schottmueleri B* 7, *Corynebacterium diphtheriae* 19, and *Vibrio cholerae* 9 (Pyatkin and Krivoshein, 1987).

Some of the bacteria mentioned are important pathogens, and since phages multiply in bacteria, it was hoped that they might afford an effective and simple way to combat bacterial diseases (Watson, 1970). Phages however have little medical use because their bacterial hosts mutate readily to forms resistant to viral growth. Furthermore, phage mutations are also as common as mutations of bacteria.

The phage-bacterium system is exploited in genetics because phages mediate bacterial gene transfer from one bacterium to another by transduction. Isolation and characterization of phages are essential because they are readily available tools for use in the dissection of important biochemical and genetic problems, such as antibiotic resistance in pathogenic bacteria. The very nature of bacteriophages, their relatively simple structure made up of a protein coat surrounding one type of nucleic acid, either DNA or RNA, and their partial independence of the cells in which they grow make them useful material for probing the molecular biology of bacteria (Hershey and Chase, 1952). They also provide an easy means of access to the nucleic acid, and this makes manipulation of genes possible in the ever expanding field of recombinant DNA technology. Studies on how phages

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act as vectors for the transfer of bacterial genetic markers such as those for drug resistance and versatility in nutrition have helped to explain the mechanism of drug action or the function of antibacterial agents.

1.1. 2 PHAGE MORPHOLOGY

Most phages have morphologies similar to the T-set coliphages, which infect *E. coli*. Some phages resemble sperm or tadpole. The T-even phages of which T4 is the most widely studied member contains DNA in its head, a tail, through which the DNA is injected into the host cell during infection and a base plate with six tail fibres, which recognize and attach to sites on the surface of the host cell (Fig. 1).

It has been observed that there are a few different types of protein in a virus. Watson and Crick, (1957), observed that the nucleic acid of small virions is probably insufficient to code for more than a few types of protein molecules. Thus small viruses comprise of identical protein subunits called capsomeres. It is these capsomeres that make up the large monomeric head or capsid of the virus (Lwoff *et. al.*, 1962). The capsomeres result in two major types of shape or symmetry, helical (screw) as in phage M13 and cubic, which may be icosahedral. T-even phages may have binal symmetry. These have combined two distinct patterns of symmetry in the head and in the tail. Larger and more complex viruses may have envelopes surrounding their heads, or specialised structures like tails. Electron micrographs have shown that

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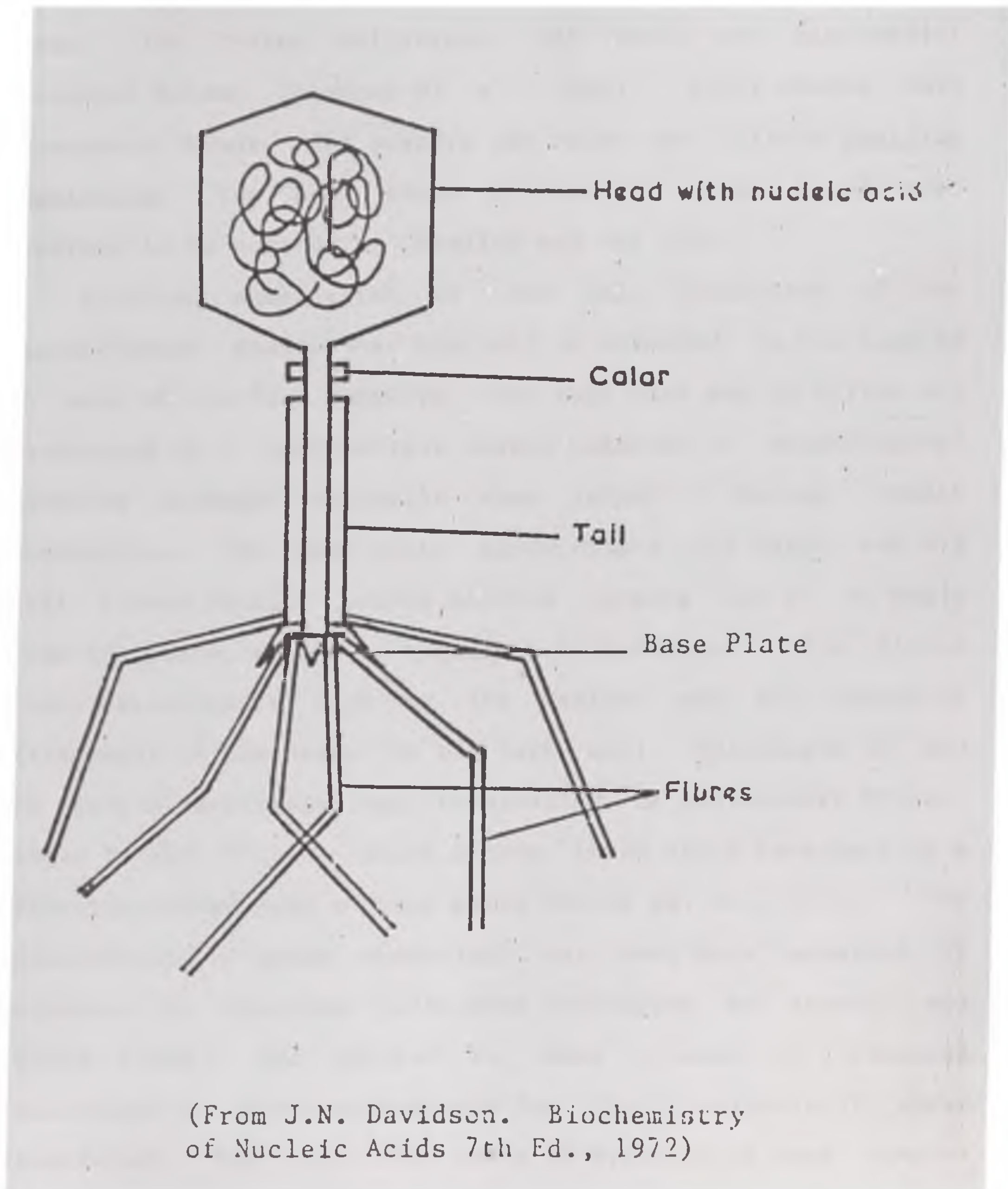

Fig. 1: The structure of a T-even phage.

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generally, different phages have heads of different geometric forms; for T-even coliphages, the heads are bipyramidal hexagonal prisms (Brenner *et. al.*, 1959). Other phages have icosahedral heads, for example the phage that infects Bacillus megaterium. The head shape of typhoid phage 2 has been reported to be octahedral (Bradley and Kay 1960).

Detailed examination of the tail structures of the bacteriophage showed that the tail is attached to the head on an axis of six-fold symmetry. The tail core may be hollow and surrounded by a contractible sheath composed of morphological subunits arranged helically when intact. During sheath contraction, the base plate moves toward the head, and six tail fibres usually become visible turning out at an angle from the plate, which is hexagonal in structure. The fibres characteristically kink at the centre, and are organs of attachment of the phage to the host cell. Coliphages T1 and T5 have a sheathless tail terminating in rudimentary fibres, while T3 and T7 have short stubby tails which terminate in a structure resembling a base plate (Davis *et. al.*, 1970). The elucidation of phage morphology has been made possible by advances in staining techniques introduced by Brenner and Horne (1959) and applied to many viruses in electron microscopy to yield micrographs for direct analysis of virus morphology. The structural basis of function of some complex viruses like the T-even phages has been determined.

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1.1. 3 PHAGE NUCLEIC ACIDS

Bacteriophages contain either DNA or RNA, but never both (Watson, 1970). The molecular weight of viral DNA ranges from 1.5 to 150kbp while for RNA it ranges from 1.5 to 15kbp.

There are three types of phage nucleic acids. These are single-stranded DNA as occurs in OX174, double-stranded DNA in T-even phages, and single-stranded RNA in MS2, R17, F2 and QB.

The type of nucleic acid may dictate phage morphology. Thus single-stranded DNA bacteriophages have been found to be either spherical or filamentous as in OX174 and fd respectively. In the filamentous shape, a circular DNA molecule is wrapped into a protein coat and formed into a filament of two nucleoprotein strands by bringing the opposite ends of the circle together. Also all RNA phages are spherical, for example MS2. DNA structure of the viruses can be determined by the extraction and analysis of the intact nucleic acid. Sinsheimer (1959) described the single-stranded circular DNA for OX174, and Espejo *et. al.*, (1969) isolated double-stranded circular DNA in supercoiled form from phage PM2. Yamagishi *et. al.*, (1965) obtained double-stranded linear DNA with cohesive ends from phage O80. Terminal Repetition was determined for phage T7 by Ritchie *et. al.*, (1967) while circular permutation was observed for phage T2 (Thomas and MacHattie, 1964).

During replication, the nucleic acid of the progeny virions may be derived from three sources, namely the nucleic acid of the infecting virions, the materials present in the host cell infected and the culture medium. According to Davis

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et. al., (1970), for T-set coliphages, the host material present during infection contributes about 30% of the phosphorous for T2 DNA and 90% for the DNA of phage T7.

Replication of phage DNA is semiconservative which means only one of the strands of each daughter DNA molecule is newly synthesised, the other is passed on unchanged from the parent DNA molecule. Since the process is accompanied by breakage and rejoining of DNA molecules, genetic recombination results. T4 has been reported to express no less than twenty enzyme activities concerned with DNA metabolism. Those enzymes engaged in synthesis of modified bases are bacteriophage specific and are usually among the early proteins synthesized during infection. In T-even phages infection is followed by the removal of CTP from the intracellular pools to prevent the incorporation of cytosine into the phage DNA. In the reproductive cycle of T4, the host cell membrane appears to be involved (Kozinski and Lin, 1965). Frankel (1966) suggested that soon after infection, parental viral DNA is attached to host cell membrane where it replicates. Very early in infection, a small amount of newly synthesized phage DNA is attached to the parental viral DNA by host enzymes (Murray and Mathews, 1969), presumably playing a role in membrane attachment. Release of progeny DNA from the membrane is a late phage function. The attachment of T7 DNA follows a similar pattern (Siegel and Summers, 1973). In normal infection the early region of T7 is transcribed by the host RNA polymerase and the late region by a T7 RNA polymerase coded by the early region (Siegel and Summers, 1973;

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Chamberlin *et. al.,* 1970).

The semiconservative replicative scheme was disputed by the finding of a single-stranded DNA in phage OX174; however this single strand was shown to last for only a part of the life cycle of the virus.

DNA viruses use many host specific proteins in their replication and genomic expression. However in the case of RNA viruses, there is a problem of uninfected host cells lacking enzymes for synthesizing RNA according to the instructions of an RNA template. The RNA viruses contain genetic information for the synthesis of an RNA-directed RNA polymerase (RNA replicase) or for an RNA-directed DNA polymerase. By designation for RNA virus, mRNA is defined as (+)RNA and its complement as (-)RNA. Replication and transcription of RNA viruses proceed by four known pathways. For example, phages R17 and QB are first class viruses known as positive strand RNA viruses. They synthesize (-)RNA which then serves as a template for the formation (+)RNA. RNA viruses possess three basic genes coding for the protein coat, replicase subunit and maturation protein. Also a fourth gene is present and it specifies a protein needed to lyse the host bacterial cell. Because the genes for protein degradation, protein coat and replicase subunit overlap, there is economy in the way sufficient information is packaged into the small genomes of the RNA viruses. The (+)RNA molecule of the virion serves both as messenger for the synthesis of the four proteins and as a template for producing many copies of (+)RNA. The replicase that synthesizes the (+) and (-)

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strands of phage RNA is highly specific for phage RNA, hence host RNA molecules do not compete with phage RNA for replication.

There have been major advances in techniques of virus isolation and characterization. Density gradient centrifugation in caesium chloride and sucrose have been widely used for purification and separation of particles with differences in nucleic acid content and composition (Cohen, 1960). Because of the length of time required for the ultracentrifugation, an alternative method, that of Yamamoto *et. al.,* (1970) is widely employed. This is a simpler purification scheme that leads to extraction of DNA. The phage preparations obtained by this method is not as pure as those obtained from density gradient centrifugation although they are clean enough for electron microscopy, sodium dodecylsulphate polyacrylamide gel electrophoresis and for agarose gel fractionation.

1.1. 4 PHAGE PROTEIN COAT

The phage particle is enveloped in a protein coat which protects its nucleic acids from nucleases in biological fluids.

The protein coat of a virus cannot be a single large molecule or an assembly of a large number of different small proteins due to the small amount of viral nucleic acid that can code for few amino acid residues. However the protein coat must be large enough to cover the entire nucleic acid and viruses, circumvent their poverty by having protein coats made

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up of one or a few kinds of protein subunits (Watson and Crick, 1957).

Phage coat proteins contain polyamines like spermine, spermidine and bis-(3-amino-propyl) amine. These basic compounds, with their multiple cationic groups, can establish multiple ionic bonds by linking together different loops to facilitate packing of the nucleic acid within a tight volume of the capsid. The secondary and tertiary structures of viral proteins are thus determined by polyvalent cations (Cohen, 1960).

The phage proteins are insoluble in buffer but may be solubilized in the presence of detergent. The proteins can be fractionated according to their molecular weights by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulphate (SDS). SDS first interacts with the positive hydrophillic groups (Burkard *et. al.*, 1972) of the protein or to specific hydrophobic receptors (Tanford, 1977) followed by less specific hydrophobic binding and eventual formation of a complex with a high ratio of SDS to protein (Nelson, 1971; Reynolds *et. al.*, 1970). This complex is thought to have a rod-like shape and other unusual hydrodynamic characteristics which account for the utility of SDS in PAGE (Reynolds *et. al.*, 1970). In this complex, the polypeptide is coated with a layer of SDS molecules at a high level of binding as well as a constant binding ratio that generally masks the negative charge of most proteins and an approximately constant negative charge per unit mass will be obtained (Sergent and Jackson, 1972). Proteins so saturated

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with detergent assume a rod-like conformation, the length of which is proportional to the polypeptide chain length and thus its molecular weight (Weber and Osborn, 1969).

Small proteins move rapidly through the gel, whereas large ones stay at the top near the point of application of the mixture. The mobility of most polypeptide chains under these conditions is linearly proportional to the logarithm of their mass, although some proteins, mostly glycoproteins and membrane proteins, do not strictly obey this rule and may produce slightly concave graphs. A calibration curve of this nature can be used to determine molecular weights of unknown proteins.

SDS-PAGE can therefore be used to determine the molecular weight of polypeptide chains of a given protein by comparing their electrophoretic mobilities on SDS-PAGE gels to the mobilities of proteins with polypeptide chains of known molecular weight. The method requires only microgram quantities of proteins, is reliable, reproducible and the results obtained in the molecular weight range of 15,000 - 200,000 daltons are generally within 10% of those obtained by other techniques (Weber and Osborn, 1969; Shapiro *et. al.*, 1967).

Phages are good antigenic materials, being capable of stimulating antibody production when introduced in an animal. The phage particles are emulsified with complete Freund's adjuvant which contains heat killed mycobacteria that have the property of enhancing antigenicity in the animal by virtue of the endotoxin they possess and the depot effects. Since the

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outer coat of the virus particle is composed of proteins, it is reasonable to suppose that the antibodies produced against the virus will be directed against these surface proteins (Hershey and Chase 1952). The serum of animals immunized with a particular virus can be assayed by determining the degree of inactivation of the homologous phage as well as other phages by the method of Clowes and Hayes (1968). The method allows one to determine whether phages are antigenically related.

1.1. 5 THE PHYSIOLOGY OF PHAGE INFECTION

Weidal *et. al.* (1954) extracted and purified a lipoglycoprotein complex which represents a minor component of the cell wall of E. coli; this protein showed a specific inactivating effect upon the E. coli phage T5 and was assumed to function in situ as receptor site for the phage. Kinetics of interaction studies between the receptor substance and T5 showed that one particle of receptor substance could combine with only one T5 particle and vice versa.

1.1. 6 ATTACHMENT AND PENETRATION

Infection begins with a random collision between phage and bacterium followed by a more precise attachment of the phage tail to receptor sites (Weidel and Kellenberger, 1955). The environment is an important factor in adsorption. The presence of inorganic salts neutralizes the net negative charges on bacterium and phage so that initial contact is facilitated. Many phages, for example P1, require Ca^{++} and lambda requires Mg^{++} for optimal adsorption whereas T4 and T6

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require L-tryptophan as an adsorption co-factor (Kellenberger *et. al.,* 1965). T2, T5 and T6 fail to adsorb to cell walls where the outer lipoprotein have been extracted with phenol, although such an extraction enhances adsorption by T3, T4 and T7.

Hershey and Chase (1952) demonstrated that only the DNA and not the protein of a phage penetrates the infected bacterium. Weidel and Primosigh (1958) provided a clue about how penetration occurs by discovering that the tail of T-even phage as well as of lambda contain a lysozyme-like enzyme which acts on the peptidoglycan layer of the cell wall, liberating alanine, glutamic acid and diaminopimelic acid.

1.1. 7 HOST CELL FUNCTION AND PHAGE GENE EXPRESSION

In T-even phage infection, which apparently is quite autonomous as regards DNA replication, host cell DNA synthesis is stopped upon infection (Tomizawa and Sunakawa, 1956) and the host DNA is degraded in parallel with phage DNA synthesis (Hershey *et. al.,* 1953). It is thought that this is accomplished by a viral coded enzyme which specifically recognizes DNA containing cytosine residues (Wiberg, 1967). The presence of new deoxyribonucleases has been reported in infections with T-even phages (Short and Koerner, 1965), lambda (Radding, 1964) and T5 (Paul and Lehman, 1966).

In the case of T5, the host cell DNA synthesis is halted and the DNA is degraded prior to viral DNA synthesis (Murray and Whitfield, 1953): In this case, a T5 coded 5′ nucleotidase converts the nucleotides to nucleosides which are then lost

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from the cell. During T7 infection, the host DNA is extensively degraded and the degradation product reutilized to make new viral DNA (Putman *et. al.*, 1952). Host DNA synthesis undergoes a peculiar kind of suppression in lambda (both lytic and lysogenic). In lytic infection, DNA synthesis is not completely halted until relatively late in infection, but in lysogenic infection, it is first depressed and then resumes at a normal rate at a later time (Smith and Levine, 1964; Sinsheimer, 1968). The de novo synthesis of DNA polymerase has been demonstrated during infection with phage T2 (Aposhian and Kornberg, 1962), T4 and T5 (De Waard *et. al.*, 1965).

1.1. 8 PHAGE ASSEMBLY AND DNA PACKAGING

Phage lambda has the head and tail assembly pathways. Assembly of the tail is controlled by a cluster of at least eleven genes(Z, U, V, G, T, H, M, L, K, I and J) and the head by another cluster of at least ten genes (Nu1, A, H, M, B, C, Nu3, D, EFI and FII). In addition, a host gene (gro E) is involved in lambda head formation (Georgopoulos *et. al.*, 1972, 1973).

The basic pattern of lambda head assembly pathway resembles that of other complex bacteriophages such as P22, T3, T5, T4, T7, P2 and P4 (Casjens and King, 1975; Hohn and Kellenberger, 1976). First, with the help of a "scaffolding protein", a prehead is assembled into which DNA is packaged, then the head is made competent for tail attachment.

Likewise, lambda tail assembly resembles that of complex phages such as T4 and involves the formation of the initiator

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for the polymerization of the major tail proteins in which most of the tail genes´ proteins are involved.

With the help of three phage packaging proteins (pNu1, pA and pFI), and "decorating protein" (pD), lambda DNA is packaged into preheads (Kaiser *et. al.,* 1975; Becker *et. al.,* 1977a, 1977b). The packaging of lambda DNA during virion assembly is coupled with the cutting of the DNA to make cohesive ends; the right cohesive end of the packaged DNA is located close to the head- tail junction. Four bases at this end can be digested in tailless heads with micrococcal nuclease, while the left end remains completely protected (Gillin and Bode, 1971, 1972). Treatment of the phage with formaldehyde and subsequent spreading for electron microscopy showed that the DNA remains attached to the head as well as to the tail; the DNA protrudes into the upper third of the tail (Thomas, 1974; Chattoraj and Inman, 1974). Several processes are coupled to the packaging; the specific selection of lambda DNA from a pool that still contains host DNA, the enlargement of the prehead by about 20% in radius, the incorporation of pD into the capsid and the processing of concatameric DNA (Hohn and Kellenberger 1976).

The conformation of DNA in isometric heads is known to consist of an ordered structure in which the double stranded molecule is wound in such a way that the majority of the sequence is present in turns around a common axis (Earnshaw and Harrison, 1977; Kostarko *et. al.,* 1979). Supercoil domains also occur in the native phage head.

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Two different proposals have been made for phage head assembly; either the DNA and the subunit proteins interact to form filled heads or empty heads are precursors of filled heads so that the DNA does not determine the geometry of the head. The latter forms the basis of the "headful" hypothesis (Streisinger *et. al.*, 1967). This model will explain the circularity of the T4 linkage map and applies equally well to P22. This model is meaningful since it derives from the fact that there is a correlation between the size of the head and the amount of DNA it contains as seems to be the case for T4. Further evidence supporting this model showed the existence of precursor heads devoid of DNA into which the viral DNA is packaged and that internal peptides may be involved in the process (Laemmli and Favre, 1973).

1.1. 9 PHAGE DNA: MOLECULAR STRUCTURE AND BASE COMPOSITION

A wide range of molecular sizes for the bacteriophage DNA molecules is found; from the small circular single stranded DNA of phage OX174 with molecular weight 1.7 x 10^6 (Sinsheimer, 1959) to the large linear double stranded DNA of complex phages such as T4 with molecular weight 108 x 10^6 (Freifelder, 1970). The size of viral DNA molecule enables an appropriate estimation of the genetic capacity of the virus. Small bacteriophage genomes may only code for bacteriophage structural proteins and control elements (for example a repressor) and may make use of host cell functions for the synthesis of progeny bacteriophage particles.

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In bacteriophage OX174, four genes are needed for capsid formation and up to six are required for other functions. Bacteriophage lambda with DNA molecular weight of 31 x 10^6 (Davidson and Szybalski, 1971) and contains no less than thirty five structural genes (Szybalski and Herkowitz, 1971) and T4 bacteriophage contains at least fifty genes involved in the production of the structural components of the phage and others with various functions (Levine, 1969). Bacteriophage P4 is unusual in that it contains a small DNA (molecular weight 7 x 10^6) and requires some of the structural proteins of phage P2, a 'helper phage' for P4 replication (Inman *et. al.*, 1971). All viruses studied to date contain not more than one copy of genome per particle. All single stranded DNA viruses have a structurally circular genome, while double stranded DNA molecules isolated from bacteriophage particles have a linear structure with the exception of phage PM2 (Espejo *et. al.*, 1969) and phage RO6P (Earnshaw and Casjens, 1980), which contain double stranded circular DNA molecule.

Some bacteriophages contain only the four normal bases; adenine (A), guanine (G), Cytosine (C) and thymine (T) in their DNA molecules, the molar percentage of these bases in the DNA varies, being similar in related phages. The bases may be segmentally distributed in a non-random fashion; in lambda DNA the average (G+C) content is 50 mole percent, but the left hand end of the molecule has on the average 56 mole per cent (Skalka *et. al.*, 1968). Such a distribution of bases may have a functional significance (Davidson and Szybalski, 1971). T4, T5, T7 and P1 DNA molecules, for example, are much

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more uniform in base composition. Some phage DNA molecules contain modified bases which may either totally or partially replace one of the normal bases. T-even phage DNA contains 5-hydroxymethyl-cytosine as a total replacement of cytosine (Wyatt and Cohen, 1953); some of these are glycosylated (Lehman and Pratt, 1960). The nature of the glycosyl side chain (alpha and beta glycosyl) as well as the relative amounts of each are different in T5, T4 and T6. A partial replacement of adenine with 6-methyl aminopurine is found in T2 and T4 DNA but not in T6 DNA (Hattman, 1970) while T3 DNA is completely unmethylated (Gefter *et. al.*, 1966). A small variable partial replacement of cytosine with 5-methylcytosine has been observed in P22 and lambda DNA molecules (Arber, 1968; Hattman, 1972).

1.1. 10 RESTRICTION AND MODIFICATION OF DNA

The processes of DNA transfer from one bacterium to another include phage infection, conjugation and uptake of free DNA. Once inside a recipient cell the DNA may survive, possibly multiply or recombine with the recipient genome, or it may be degraded by a process known as restriction. The survival or restriction of foreign DNA depends upon the restriction specificity of the recipient cell and upon certain nonheritable characteristics imparted to the DNA by the cell from which it came. These characteristics are called host-controlled modifications.

Although the phenomenon of host-controlled restriction and modification has been observed many years previously

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(d'Herelle, 1926; Graigie and Yen, 1938), a systematic study was begun in the 1950's (Luria and Human, 1952; Bertani and Weigle, 1953). They investigated structures in which the ability of a phage to plate efficiently on a given bacterial strain depended on the strain on which the phage was last propagated. When a phage is transferred from one host strain to another the viral titres on the two strains may differ considerably; if the surviving phages are used to infect a culture of the second host strain they grow quite normally, but when passed through their original host strain their efficiency of growth is impaired once again. There are also strains such as E. coli that appear to be without any restriction - modification system.

Restriction enzymes are endodeoxyribonucleases that recognise specific nucleotide sequences in double stranded DNA and cleave both strands of the duplex. In the cell of origin, each restriction endonuclease is associated with a matched modification system which recognises and modifies (generally by methylation) the same nucleotide sequence in DNA recognised by the restriction enzyme (Arber and Lin, 1969; Kuhnlein and Aber, 1972; Smith *et. al.,* 1972). Such restriction - modification systems are widespread in bacteria and may play a role in eliminating foreign DNA that gains entrance to the cell via viruses or as naked DNA. The two activities compete so that a certain proportion of infecting DNA is protected from degradation by the restricting activity.

Restriction enzymes may be placed into two classes; not all of them cleave DNA at specific sites although all appear

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to recognize specific nucleotide sequences. The class I enzymes are non specific in their cleavage (Boyer, 1971; Arber, 1974) and have the following properties; molecular weight of about 300,000 (Meselson and Yuan, 1988), non identical subunits (Meselson *et. al.*, 1972), require ATP, Mg^{++} and S-adenosylmethionine (SAM) as cofactors and do not produce molar yield of specific fragments. Examples are Eco B and Eco K (Meselson and Yuan, 1968).

The class II enzymes are specific in their cleavage (Boyer, 1971). These have lower molecular weight of 20,000 - 100,000, require only Mg^{++} as cofactor (Smith and Wilcox, 1970) and cleave DNA at the enzyme recognition sequence to produce specific fragments. For example Eco RI which contains two identical subunits (Green *et. al.*, 1974).

It has been possible with many of these enzymes to determine the sequence of base around the cleavage site. The first report of such an experiment was that of Kelly and Smith (1970) who determined the sequence around the cleavage sites on T7 DNA of the Haemophilus influenzae endonuclease isolated by Smith and Wilcox (1970). The enzyme isolated by Smith and Wilcox is a mixture of two endonucleases Hind II and Hind III (Old *et. al.*, 1975). Only Hind II, however, cleaves T7 DNA.

Eco RI makes "staggered" breaks four base pairs apart and the sequence is rotationally symmetrical with its axis through the centre of the four base pairs (Hedgpeth *et. al.*, 1972). In addition the six fragments released from wild type lambda DNA by Eco RI specific cleavage have been ordered by comparing the fragmentation patterns of a series of lambda derivatives

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and the cleavage sites within the genome located (Allet *et. al.*, 1973; Thomas and Davis, 1975). The practical application of restriction and modification of DNA, once the mechanisms of its function are understood are numerous: The cleavage of double stranded DNA molecules at specific sites generating a population of unique fragments or restriction fragment length polymorphs (RFLP), opens an important approach to DNA sequence determination as well as structural studies and characterization of bacteriophages and other small DNA molecules.

Fractionation of cleavage products is achieved by the use of gel electrophoresis. By choosing the appropriate gel, it is possible to fractionate DNA fragments ranging from a few hundred nucleotide pairs up to at least 30,000 nucleotide pairs (Gilbert and Maxam, 1972; Helling *et. al.*, 1974). Polyacrylamide gels are suitable for fractionation in the low molecular weight range, whereas agarose gels permit separation of larger molecules. Ethidium bromide is widely used as a fluorescent stain. It may be excited with either short or long wavelength ultraviolet light. Distinctive patterns have been obtained in this way for SV40 DNA with the H. influenzae endonucleases Hind II and Hind III (Danna and Nathans, 1971), for OX174 DNA (Edgell *et. al.*, 1973), of various lambdoid phage using Eco RI (Allet *et. al.*, 1973; Murray and Murray, 1974), and Eco RII (Bigger *et. al.*, 1973).

One striking observation is that many enzymes from different sources recognise the same nucleotide sequence; for example, restriction endonucleases with GGCC specificities

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have been isolated from Bacillus subtilis, H. aegyptus and H. hemoglobinophilus. Roberts (1975) has coined the term isoschizomers to describe such enzymes.

One use of restriction endonucleases is the physical mapping of chromosomes. This has been exploited in the case of viral chromosomes consisting of duplex DNA. Specific cleavage sites or fragments serve as physical references in the map. By analogy with tryptic or chymotryptic maps of proteins, the electrophoretic pattern of restriction fragments of DNA can serve as a fingerprint of the DNA (Nathans and Danna, 1972). Owing to the sensitivity of electrophoretic separation of fragments in appropriate gels, even relatively minor differences between similar DNAs may be detected. In molecular biology experiments, restricted DNA fragments are inserted into plasmids, phages or other vectors and the recombinant molecules are used to infect bacteria in which they replicate and express their genes. The availability of specific DNA fragments has greatly simplified nucleotide sequence analysis of DNA molecules. Restriction enzymes are important in the characterization of phage genomes. For example, Hind III-restricted lambda DNA and Eco RI-restricted lambda DNA of known molecular weights are run along with restriction fragment length polymorphs of the experimental samples generated using the same enzymes on agarose gel electrophoresis. The relative mobilities of the DNA markers and polymorphs are compared and if the fragments move the same relative distance then they are the same, however if they move

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at different rates there is a genetic difference between the samples.

1.1. 11 TEMPERATE AND VIRULENT BACTERIOPHAGES. LYSOGENY AND TRANSDUCTION

There are two general categories of phages. Virulent phage break open and destroy every bacterium they infect, having first multiplied using their host's protein synthesizing system. Temperate phages permit some of the bacteria they infect to survive the infection. These bacterial cells continue to contain the phage genetic material but under certain conditions, such as exposure to ultra violet light or to some antibiotics for example mitomycin C, the phages become active, and having multiplied, destroy their hosts by lysis of the cell walls. Bacteria carrying temperate phage are said to be lysogenic as against those carrying virulent phage, which are called lytic (lwoff, 1953).

The best studied phage is lambda; it comprises a duplex DNA of about 46.5kbp. Two options are open to lambda upon infection of *E. coli*. The lambda DNA may behave in a lytic manner where phage genes are expressed and phage DNA replicated to produce many more phage particles, which are eventually released by cell lysis. Alternatively, lambda may exist in harmony with its host cell in the non infectious manner known as lysogeny. Lysogenic bacteria carry phage DNA in a form known as prophage. By virtue of their possession of prophage, lysogenic bacteria are immune to superinfection by other phage particles of the same type (Bertani, 1953). The prophage is inherited in the same manner as bacteria genes.

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The choice of whether a phage enters a lytic cycle or forms a lysogen depends on the conditions of infection and the genotypes of phage and bacterium. One temperate phage, coliphage P2, is said to be non-inducible; virions are released spontaneously by only a small number of cells. Every cell in a lysogenic culture can lyse and liberate phages, yet if the cells are broken open, neither can phage be extracted from them nor can phage be seen under the electron microscope. Phage proteins cannot also be detected serologically. Lysogenic cultures thus behave as if they contained a gene for phage production, a gene that is normally quiescent, functioning only occasionally as if by accident or in response to specific stimuli. Lysogeny is not limited to the carriage of a single type of phage by each bacterium; many cases of double and triple lysogeny have been described, and one strain of Staphylococcus has been reported to carry as many as five different phage types (Rountree, 1949; Rountree and Asheshov, 1961; Duval-Iflah, 1972). However, lysogeny should be judged by rather rigorous criteria for its stability, since the interaction of some virulent phages with their hosts may superficially stimulate the condition. A population of sensitive bacteria exposed to a very low concentration of virulent phage, after initial infection will have difficulty in reinfecting new cells. An equilibrium will be thus established in the population between uninfected cells and free phage particles. On subculture however, the phenotypically resistant bacteria become sensitive again and the process is repeated. This type of relationship is found

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when T7 infects Sh. dysenteriae. Apparently lysis of the initially infected bacteria releases an enzyme which removes the phage receptors from the cell surface of the remaining uninfected bacteria, rendering them resistant until the enzyme is diluted out of the subculture (Koibong *et. al.,* 1961).

Bacteriophages can mediate the transfer of bacterial genes from one bacterium to another, a process called transduction. Transduction was first noted by Lederberg *et. al.,* (1951) who mixed two auxotrophic strains of S. typhimurium unable individually to grow on minimal medium and noted that prototrophic recombinants appeared not only when cultures of two Salmonella strains were mixed but also when a culture of one strain was treated with a cell free filtrate of the other. Zinder and Lederberg (1952) soon recognized that the agent in the filtrate was the phage P22. The occurrence of transduction in bacteria and phages has since been reported in genera as diverse as Escherichia phages lambda, P1 and 363 (Morse, 1954; Lennox, 1955), Shigella phage P1 (Lennox 1955; Adams and Luria, 1958), Pseudomonas (Loutit, 1958), Staphylococcus (Morse, 1959), Proteus (Coetzee and Sacks, 1960), Bacillus, Rhizobium and Caulibacter. When a phage lysate is prepared from a donor bacterium, only a small fraction of the progeny particles (10^{-5} - 10^{-7}) can transmit genetic characteristics to a subsequent host.

Transducing phages can be divided into two classes on the basis of the host markers which they are able to transfer: The P22/Salmonella system is a characteristic example of generalised transduction, where any small region of the donor

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chromosome can be transduced. An unusual variant of P22, called P22 TC 10, transduces tetracycline resistance at high frequency (Watanabe *et. al.*, 1972; Chan *et. al.*, 1972). Examination of P22 TC 10 DNA under the electron microscope showed that this specialized transducing variant contains a large (8.3 kilobase) insertion with an unusual structure; it consists of an inverted duplication separated by non repeated DNA sequences. The tetracycline resistance insertion was acquired by P22 during a lytic cycle of growth in a Salmonella strain harbouring a drug resistance plasmid in whose DNA a similar reverse duplication was found to be associated with the genetic determinant of tetracycline resistance.

Restricted or localized transduction, on the other hand, is limited naturally to phage that can lysogenise bacteria: Transfer of chromosomal genes is restricted to a cluster of genes adjacent to the prophage location on the bacterial chromosome. This kind of transduction is exhibited only with lysates produced by induction of the prophage. Transduction by lambda represents the best defined specialized system (Morse *et. al.*, 1969, b). Phage lambda, carrying the gal genes, was prepared by inducing gal^+ lambda lysogens, isolating the phage particles, mixing the phage particles with gal^- bacteria and isolating gal^+ transductants. Such a process yields one transducing particle in 10^6 and is called low frequency transduction (l.f.t.). It is however, possible to induce gal^-/ lambda gal^+ heterogenotes to obtain a high frequency transduction (h.f.t.). On induction, lambda prophage, occasionally undergoes exchange with the nearby gal

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segment of the bacterial chromosome. Although the particles (lambda d gal) which result are defective because essential lambda genes are excluded , they are able to transduce the gal region.

Transduction is a valuable tool in mapping out the bacterial chromosome. Its use in fine structure mapping is analogous to mapping by transformation; although it does not suffer from the problems of spurious linkage since only a small proportion of the phage lysate is transducing, in contrast to the potential activity of every DNA molecule in a transforming preparation. The degree of linkage of genetic markers can be inferred from the relative frequency with which they are co-transduced.

1.1. 12 BACTERIAL-PHAGE RELATIONSHIP

The use of bacteriophage is governed by the ease with which the bacteria they infect can be cultivated. Thus, the most extensive studies are of the viruses that infect E. coli or S. typhimurium. These are the virulent T phage, and the temperate lambda and P22 phage, each of which has double-stranded DNA. Also well known, are the single-stranded DNA phage OX174 and RNA phages MS2 and QB.

Propagation of millions of phage particles in a very short time is technically simple. They are grown in the laboratory by inoculating a culture of bacteria with the phage. As the phage reproduces itself, it lyses the bacterium it has infected and its offspring infect other bacteria. This cycle continues until a maximum concentration of virus

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particles is reached. The inoculum may be prepared by a mixture of phage sample and bacteria stock solution which is then spread out on top of a solid medium (contained in a petri dish) on which the host bacteria will grow. After incubation, the bacteria form a dense layer over the surface of the petri dish. However, where a bacteriophage was present, it would have lysed its host bacterium. Its offspring would have infected and killed surrounding bacteria thus creating clear areas called plaques in the layer (lawn) of bacteria. Also the concentration of phage in the stock solution may be estimated by the number of plaques that appear in the layer of bacteria.

1.1. 13 OBJECTIVES

In this project the relatedness of bacteriophages within the Legon-Tema communities was studied, and their similarity or otherwise compared to already known bacteriophages. The bacteriophages were first isolated using indicator bacteria strains (clinical isolates from the Korle-Bu teaching hospital and the Noguchi Memorial Institute for Medical Research). It was observed whether bacteriophages from these communities, could infect such clinical isolates with a high frequency.

The phages were then propagated and purified using the alternative method of Yamamoto *et. al.*, (1970), that also leads to the extraction of viral DNA. The availability of DNA from these phages, could provide the basis for the possibility of using some of the phages in recombinant DNA and transformation experiments.

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The phages were partially characterised using the following parameters; phage morphology using electron microscopy with emphasis placed on head measurements; antigenic relatedness using immunological studies, that is by raising antisera against any of the phage particles and using it to inactivate other phage particle; DNA fingerprinting; by restriction fragment length polymorphism of Eco RI and Hind III-restricted phage DNA comparison using agarose gel electrophoresis; and protein profiles in which phage polypeptide bands run alongside standard proteins using SDS-PAGE were compared.

Some of the phages were screened for their ability to transduce antibiotic resistance genes among bacteria. The criterion was, to first obtain the minimum antibiotic inhibitory concentration (MIC) values of all the clinical isolates using agar dilution. Further increased tolerance levels of the respective antibiotics by the clinical isolates was induced using training in which the antibiotic tolerance of the clinical isolates were raised stepwise by small increments from just below the MIC values to relatively very high concentrations until there was no cell growth. Some of the phages were then used to transduce antibiotic resistance genes from the resistant strain of bacteria to the sensitive strain.

In an unrelated work , the discovery of drug resistance of certain pathogenic bacteria to antibiotics during routine clinical laboratory tests (Bryant, 1972) enabled the

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comparison of the MIC values of the clinical isolates to their induced increased tolerance levels.

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CHAPTER 2

2.1 MATERIALS AND METHODS

2.1. 1 MATERIALS

Nutrient broth was obtained from E. MERCK DARMSTADT GERMANY. The following chemicals were obtained from FLUKA AG, BUCHS SWITZERLAND: Nutrient agar, Sodium chloride, Sucrose, Potassium hydroxide, Magnesium sulphate ($MgSO_4 .7H_2O$) analar grade, Calcium chloride, Disodium hydrogen phosphate, Sodium dodecylsulphate (SDS), Ammonium persulphate, Hydrochloric acid, 2-mercapto-ethanol, Tris-(hydroxymethyl)-aminomethane, Acrylamide, Bromophenol blue, Coomasie brilliant blue R-250, antibiotics (Tetracycline, Penicillin-V-sulfoxide and Erythromycin). Glacial acetic acid analar grade, Polyethylene glycol (PEG 6000), Ethylenediamine tetra-acetic acid tetrasodium salt (EDTA) and Potassium hydrogen phosphate were from HOPKIN AND WILLIAMS, CHADWELL HEATH ESSEX ENGLAND. Glycine, Potassium acetate, Boric acid and Isopropanol were from BDH CHEMICALS LTD., POOLE ENGLAND. NN′-methylene bis-acrylamide was from EASTMAN KODAK COMPANY, ROCHESTER, N.Y., USA and all the photographic material was from FUJI FILM CO, TOKYO, JAPAN and KODAK INTERNATIONAL BIOTECHNOLOGIES INC. NEW HAVEN USA. Chloroform was supplied by ALDRICH CHEMICAL CO. LTD., GILLINGTON, DORSET, ENGLAND. The molecular weight markers for Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) were from PHARMACIA BIOTECH. EUROPE. BRUSSELS, BELGIUM. Restriction endonucleases Eco RI

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and *Hind III* were supplied by SIGMA CHEMICAL CO., ST LOUIS, USA. Ethidium bromide and Agarose were supplied by BETHESDA RESEARCH LABORATORIES GAITHERSBURG USA. Freund's adjuvant complete and incomplete were obtained from IATRON LABORATORIES CHIYODA-KU, TOKYO JAPAN.

2.1. 2 SOURCES OF BACTERIAL STRAINS

Eleven bacterial strains were used for the project work. They are; *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Salmonella typhi*, and *Shigella flexneri* from the bacteriology unit of the Noguchi Memorial Institute for Medical Research (NMIMR), Legon and *Salmonella group D*, *Klebsiella species*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, and *Proteus species* from the Microbiology Department, University of Ghana Medical School Korle-Bu.

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2.2 METHODS

2.2. 1 MAINTENANCE OF BACTERIAL CULTURES

Bacteria and phages were handled under aseptic conditions. Maintenance of the bacteria strains was by serial transfer in liquid culture or on solid media. The strains were grown in nutrient broth 2.5% (w/v). The indicator cultures, up to about 100.0ml, were grown in 250.0ml Ehlenmeyer flasks on a shaker at room temperature, plated on solid media containing approximately half- full petri dish of 1.3% (w/v) nutrient agar and 2.5% (w/v) nutrient broth. Nutrient broth (2.5%) was used for the dilution of bacteriophages.

2.2. 2 ISOLATION OF BACTERIOPHAGES

Nutrient broth was added to the suspected solid phage samples and these, together with the liquid phage samples (source table 1), were filtered through Whatman Number 4 filter paper into clean test tubes. A few drops of chloroform were added to each filtrate which was further filtered through a sterile bacteriological filter (mesh size 0.2µm) into sterile tubes. The samples were then stored at 4°C.

0.1ml aliquots of the suspected phage filtrates were pipetted into tubes containing 1.0ml of indicator bacterial cells. To these 2.0ml of molten overlay sloppy agar (containing 0.65% w/v nutrient agar, 2.5% w/v nutrient broth, 1.0mM $CaCl_2$ and 1.0mM $MgSO_4$) were added. The mixture was then poured on to solid media (prepared as in 2.b.1). When the

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overlay agar set, the plates were inverted and kept at 37°C in an incubator overnight, or longer. The plates were then examined for the presence of phage. Plates were prepared in duplicate for each suspected phage filtrate and the presence of phages in a sample was detected as plaques on lawns of the indicator host bacterial cells.

Sterile toothpicks were used to remove the plaques which were transferred directly into a sterile screw cap bottle containing 15.0ml sterile 2.5% (w/v) nutrient broth and 6 drops chloroform, and stored at 4°C. Where plaques were too numerous to be handled conveniently with a toothpick, the overlay agar was scrapped off and transferred into a sterile beaker. About 20.0-25.0ml of 2.5% (w/v) nutrient broth containing 0.5ml chloroform was added and the mixture was homogenized in a WARING BLENDOR. The debris was removed centrifugation using the HITACHI 20PR-52D high speed centrifuge, with rotor number RPR20-2-1128 at 16,000 rpm at 4°C for 20 minutes.

2.2. 3 PROPAGATION OF BACTERIOPHAGES

Bacteriophages were titred, and the dilution with the appropriate phage concentration (confluent lysis or numerous plaques on the indicator bacterial cell lawn) were propagated on 100 solid media plates. To these plates were added 0.1ml aliquots of the appropriate phage titres plus 1.0ml overnight culture of indicator bacterial cells and 2.0ml molten overlay sloppy agar mixtures. After the overlay agar had set the plates were incubated overnight at 37°C. The overlay was

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scrapped off from each plate with the flat end of a sterile spatula into a 500.0ml beaker. About 250.0ml 2.5% (w/v) nutrient broth and 20.0ml chloroform were added and the mixture homogenized. The indicator bacterial cell debris was removed by centrifugation using the HITACHI 20PR-52D high speed centrifuge, with rotor number RPR20-2-1128 at 16,000 rpm at 4°C for 20 minutes. The supernatants were decanted and stored at 4°C.

2.2. 4 TITRATION OF BACTERIOPHAGES

To determine the titre of the phage stock, serial dilutions were prepared. Aliquots (0.1ml) of the dilutions of phage preparations were plated out on indicator bacteria.

2.2. 5 PURIFICATION OF BACTERIOPHAGES

Bacteriophages for *Sh. dysenteriae*, *S. typhi*, *E. coli* and *S. group D* were purified according to the modified method of Yamamoto *et. al.*, (1970). Suspensions of bacteriophages were used.

To 50.0ml each of phage suspension were added solid sodium chloride to a final concentration of 1.0M, dissolved by gentle swirling, and the samples left to stand for one hour on ice. The debris was removed by centrifugation using the TOMY CX 250 high speed centrifuge at 11,000g at 4°C for 10 minutes. The supernatants were decanted into clean beakers. Solid polyethylene glycol was added to the supernatants to a final concentration of 10% (w/v). The samples were kept on ice for about an hour. The precipitates were recovered by

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centrifugation at 11,000g at 4°C for 10 minutes. The supernatants were discarded and the fluids that were left were completely drained away from the pellets. The bacteriophage particles were resuspended in 0.8ml of sterile storage medium, SM (5.8g NaCl; 2.0g $MgSO4.7H_2O$; 50.0ml of 1.0M Tris HCl; pH 7.5 and 5.0ml of gelatin to 1 litre H_2O) for each 50.0ml of supernatant. The walls of the centrifuge tube were well washed to prevent bacteriophage particles sticking to them.

An equal volume of chloroform was added to the bacteriophage suspension. The organic and aqueous phases were separated by centrifugation using the TOMY CX 250 high speed centrifuge at 16,000g at 4°C for 15 minutes. The bacteriophages were recovered from the aqueous phases and collected by centrifugation using the HITACHI 80P-7 ultracentrifuge, with rotor number RP50T-249 at 25,000 rpm at 4°C for 2 hours. The supernatants were then poured off leaving slimy (glassy) pellets; these were resuspended in 2.0ml SM.

2.2. 6 CHARACTERIZATION OF BACTERIOPHAGES

Bacteriophages were characterized by electron microscopy, immunological studies, DNA restriction fragment length polymorphism, protein profile and transducing ability

2.2. 7 ELECTRON MICROSCOPY OF BACTERIOPHAGES

The method of Brenner and Horne (1959) was employed in the preparation of phage specimens for electron microscopy: The copper grids were covered with a collodion film.

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Evaporated carbon was then used to coat the film. A drop of purified phage suspension was then placed on coated copper grids from finely drawn glass pipettes. Excess bacteriophage solution on the grid was blotted away. A drop of stain, 2% uranyl acetate, was applied onto the grids; it was left for 45 seconds and the excess stain was blotted away, The grids were left to air dry. The stained grids were then observed under the HITACHI H6000 transmission electron microscope, at an acceleration voltage of 75KV. The images were photographed at magnifications of 120,000 and 160,000.

Images (micrographs) were documented on a FUJI FC orthochromatic electron microscope film. Films were developed for 4 minutes in a FUJI copinal microfilm developer, rinsed in FUJI stopper for 1 minute, and fixed for 8 minutes in FUJI fixer film and then washed in running water for 10 minutes; dripped in driwel and finally dried in a film oven at 65°C.

2.2. 8 BACTERIOPHAGE ANTISERA PREPARATION

Antisera against bacteriophages StRCL16 and EcRCL24 were raised in two strains of four month old New Zealand male rabbits, obtained from the NMIMR, by intramuscular injection with purified phages. The protein concentrations of the phage preparations were estimated by the Folin-Lowry method (Lowry *et. al.,* 1951). Each rabbit was injected with 150.0µg antigenic material emulsified in 0.5ml complete Freund's adjuvant. After 12 days from the initial injection the process was repeated using 150.0µg antigenic material. The rabbits were, subcutaneously given a second booster dose after

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two months, using 150.0µg antigenic material in an emulsion with incomplete Freund's adjuvant. Ten days after the last injection the rabbits were bled from the back of the ear by venous puncture. Some blood was also taken from the rabbits and screened for possible polyclonal antibody activity before the initial injections. The blood taken by venous puncture was left at room temperature for several hours and then overnight at 4°C. The blood clot was separated by centrifugation using a microfuge at 10,000 rpm for 5 minutes and the sera obtained screened for antisera activity.

2.2. 9 BACTERIOPHAGE INACTIVATION

The antisera were titred by phage inactivation (Clowes and Hayes, 1968). Forty plates of solid media were prepared. The plates were divided into two sets of 20 each. To half of one set was added 0.1ml aliquots of the appropriate phage preparations plus 1.0ml indicator bacteria and 2.0ml molten overlay sloppy agar, this corresponded to the original phage concentration (Co) in p.f.u./ml. To the other half was added all the above and also one drop of Anti StRCL16 serum diluted twenty-five times which will correspond to phage concentration (C) after t minutes incubation. The whole procedure was repeated using Anti EcRCL24 serum. After the overlay agar had set the plates were incubated overnight at 37°C and the plaques counted.

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2.2. 10 ISOLATION OF DNA FROM BACTERIOPHAGES

DNA was isolated from bacteriophages for Sh. dysenteriae, S. typhi, E. coli, and S. group D according to the method of Wilson *et. al.,* (1990). Bacteriophages were purified as described in section 2.b. 5 to obtain glassy pellets. Phage samples (1000.0µg) were each homogenized in 100.0µl of Bender buffer (0.1M NaCl, 0.2M sucrose, 0.5% SDS, 0.05M EDTA and 0.1M Tris-HCl, pH 7.6). The phage samples in the Bender buffer were incubated at 65°C for 30 minutes. Pre-chilled 8.0M potassium acetate (15.0µl) was added to each tube, mixed well and left on ice for 45 minutes. The contents of the tubes were spun down in a microfuge for 5 minutes. The supernatants were added to 2 volumes of absolute ethanol in 1.5ml Eppendorf tubes, mixed well and left to stand at room temperature for 5 minutes. The contents of the tubes were then spun down in the microfuge for 10 minutes at 28°C or room temperature. The ethanol was decanted and 100.0µl of a dilution of one hundred times TE buffer (1.0M Tris-HCl, pH 8.8 containing 0.1M EDTA) added to redissolve pellets and incubated at room temperature for 30 minutes. 5.0M NaCl (5.0µl) and 210.0µl absolute ethanol were added to each tube and stored overnight at -20°C. The contents of the tubes were then spun down in a microfuge for 10 minutes at room temperature. The ethanol was decanted and the pellets dried under vacuum for 10 minutes. TE buffer (15.0µl) was added to each tube to redissolve DNA and the samples stored until ready for use.

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2.2. 11 RESTRICTION OF BACTERIOPHAGE DNA

DNA from bacteriophages for Sh. dysenteriae, E. coli. S. typhi. S, group D and lambda DNA were digested separately with Hind III and Eco RI endonucleases. Each restriction mixture contained 0.5µg DNA, 1.0µl of ten times concentration reaction buffer (50.0mM Tris-HCl, pH 8.0; 10.0mM $MgCl_2$; and 100.0mM NaCl) and 1.0µl endonuclease in a total volume of 10.0µl. The reaction mixtures were incubated at 37°C overnight.

2.2. 12 AGAROSE GEL ELECTROPHORESIS OF BACTERIOPHAGE DNA

The Eco RI and Hind III restriction endonuclease generated fragments of bacteriophage DNA preparations and lambda DNA were fractionated by electrophoresis on 1.2% agarose gel. The gel contained 1.2g agarose, 0.045M Tris-borate, pH 8.0, 1.0mM EDTA and ethidium bromide 10.0mg/ml in a total volume of 100.0ml. The mixture was heated until the agarose had dissolved. The molten mixture was then poured at 80°C to prepare gel slabs in a horizontal gel cell. Loading buffer five times concentration (0.25% bromophenol blue and xylene cyanol: 40% w/v sucrose) was added in aliquots of 2.5µl per 10.0µl reaction mixture and shaken to mix completely. Each of the reaction mixtures (10.0µl) was loaded into the agarose gel wells. The electrode (running) buffer contained 0.045M Tris-borate, 1.0mM EDTA, pH 8.8 and ethidium bromide 10.0mg/ml in a total volume of 100.0ml. The electrophoresis was run for 1 hour at 100volts and a constant current of 50mA. DNA bands were visualized under ultra violet light using the ULTRA-LUM (Helling *et. al.,* 1974). Photographs were taken

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with KODAK instant camera and hood 0.85X DS. H.5 using polaroid film type 667 with focus 4.5 and exposure 1 second.

2.2. 13 SOLUBILIZATION OF BACTERIOPHAGE PROTEINS

0.1mg of bacteriophage were resuspended in 0.5ml of 50.0mM Tris-HCl, pH 7.0, containing 2% SDS, 5% mercaptoethanol and 0.05mg/ml bromophenol blue. The samples were mixed and incubated in boiling water for 2 minutes to dissociate the metastable protein aggregates, left to cool and stored at 4°C.

2.2. 14 SDS-PAGE OF BACTERIOPHAGE PROTEINS

The solubilized proteins of bacteriophage preparations were resolved by electrophoresis on sodium dodecylsulphate polyacrylamide gel by the discontinuous method of Laemmli (1970) which was slightly modified. The main gel contained 3.0M Tris-HCl, pH 8.8, acrylamide NN′-methylene-bisacrylamide (30: 0.8), 2% (w/v) ammonium persulphate, 10% SDS and 15.0μl Temed in a total volume of 30.5ml and the stacking gel contained 0.47M Tris-HCl, pH 5.8, acrylamide NN′-methylene-bisacrylamide (30:0.8), 2% (w/v) ammonium persulphate, 10% SDS and 20.0μl Temed in a total volume of 39.8ml. The gels were loaded into the hollow glass tubes such that 10.0cm of the main gel and 1.5cm of the stacking gel were formed. The glass tubes had an inside diameter of 5.0mm. The electrode (running) buffer was composed of 0.25M Tris-HCl pH 8.5, 0.192M glycine and 0.1% SDS.

Solubilized proteins of the 10 bacteriophage samples in 50.0μl aliquots were analyzed by electrophoresis at a constant

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current of 3mA/tube using a SHANDON Vokam 400 power pack and trough, until the tracking dye (bromophenol blue) reached about 1.0cm to the bottom of the gel. The gels were removed from the tubes and stained for protein with a staining solution (Coomasie brilliant blue, 0.18g; glacial acetic acid, 100.0ml; isopropanol, 248.0ml in 1.0 litre distilled H_2O). The gels were destained in three changes of destaining solution (glacial acetic acid, 10% w/v; and isopropanol, 5% w/v). The apparent molecular weights of the bacteriophage proteins were determined by concurrent electrophoresis of standard protein markers. The markers used were ferritin (18.5kd), lactate dehydrogenase (36.0kd), catalase (64.0kd), and thyroglobulin (330.0kd).

2.2. 15 DETERMINATION OF MINIMUM ANTIBIOTIC INHIBITORY CONCENTRATIONS (MIC) OF THE BACTERIAL STRAINS

The antibiotics tetracycline, penicillin-V-sulfoxide and erythromycin were used for the MIC tests. MIC of the bacterial strains were determined by the agar dilution method (Lawrence and Francis, 1971), in which 100.0ml each of 1.3% (w/v) nutrient agar and 2.5% (w/v) nutrient broth were prepared for ten 250.0ml Ehlenmeyer flasks. They were then sterilized at 121°C for 30 minutes in an OSK-8869E autoclave.

Stock solutions (2000.0µg/ml) of the above antibiotics were prepared. The solid media in molten form, were used to prepare serial dilutions of the stock antibiotic solutions of concentrations 0.0-200.0µg/ml. Plates were poured such that, there were three sets of 20 plates, each containing the dilution range of the three antibiotics used. The plates were

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allowed allowed to set. Overnight bacteria cultures, of the eleven bacterial strains in aliquots of 50.0µl were transferred to each plate and spread uniformly with a glass spreader. The plates were incubated at 37°C overnight and the colonies counted after optimal growth depending on their mean generation.

2.2. 16 INDUCTION OF INCREASED ANTIBIOTIC TOLERANCE IN BACTERIAL STRAINS

Increased antibiotic tolerance in the bacterial strains was induced by the "training" method (Buchard and Parish, 1975a). This technique involves exposing the sensitive bacterial strain to gradual increases of the appropriate antibiotic concentration such that the concentration is raised by small steps to allow the organism to mutate and become adapted. Plates with concentrations of the appropriate antibiotic (tetracycline, penicillin-V-sulfoxide or erythromycin) at 10.0µg/ml below the MIC values were prepared. The plates were incubated overnight at 37°C to check for sterility. Aliquots (50.0µl) of overnight cultures of the bacterial strains were added to the plates and incubated overnight at 37°C. Colonies of bacterial cells on these plates were then inoculated into liquid media (2.5% w/v nutrient broth) containing the appropriate antibiotic at the same concentrations as those of the solid media from which the colonies were transferred. Plates were then prepared with concentrations of antibiotic at 10.0µg/ml higher and 50.0µl aliquots of the overnight bacterial cultures containing the antibiotics added. Subsequent transfer of 50.0µl of the

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bacterial cultures of antibiotic concentrations parallel to plates of antibiotic concentrations that were increased by small steps of 10.0µg/ml, resulted in the formation of strains that were tolerant to very high concentrations of antibiotic. For example, in experiments involving Sh. dysenteriae, plates with a concentration of 115.0µg/ml tetracycline, 115.0µg/ml penicillin-V-sulfoxide and 10.0µg/ml erythromycin were prepared and 50.0µl aliquots of overnight bacterial cultures added to each plate, and incubated overnight at 37°C. Colonies from these preparations were then inoculated into liquid media containing the appropriate antibiotics at the same concentrations to prepare overnight cultures. Plates were again prepared, this time with concentrations of 125.0µg/ml tetracycline, 125.0µg/ml penicillin-V-sulfoxide and 20.0µg/ml erythromycin and aliquots of 50.0µl of the overnight cultures added. The process was repeated several times with the concentrations of antibiotics only raised by 10.0µg/ml each time until at final antibiotic concentrations of 220.0µg/ml tetracycline, 350.0µg/ml penicillin-V-sulfoxide and 220.0µg/ml of erythromycin there were no visible growths of bacterial cell colonies.

2.2. 17 DETERMINATION OF TRANSDUCING ABILITY OF BACTERIOPHAGES

Some bacteriophages for Sh. dysenteriae, S. typhi and E. coli were tested for their ability to transduce tetracycline, penicillin-V-sulfoxide and erythromycin resistance by a modified method of Campos *et. al.*, (1978). Bacteriophages were propagated on an appropriate resistant bacteria and added

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to sensitive recipient cells. The infected cells were incubated for 12 hours at 30°C. Samples of infected cells (0.1ml) were plated on solid media containing the appropriate antibiotic. Plates were incubated for 3 weeks before scoring for transductants. A control experiment was set up by propagating bacteriophages on sensitive cells and uninfected cells plated on the antibiotic media to make sure that the transductants that were scored were due to the transducing experiments. For example bacteriophages for Sh. dysenteriae were propagated on the bacteria cells tolerant to antibiotic concentrations of 210.0µg/ml tetracycline, 340.0µg/ml penicillin-V-sulfoxide and 210.0µg/ml of erythromycin and 25.0µl aliquots added to the respective sensitive bacteria cells. The infected cells were incubated for 12 hours and 0.1ml aliquots were plated on solid media containing 210.0µg/ml tetracycline, 340.0µg/ml penicillin-V-sulfoxide and 210.0µg/ml erythromycin and incubated for three weeks before scoring transductants. The transductants were estimated for each antibiotic and used in determining the transduction frequencies.

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CHAPTER 3

3.1 RESULTS

3.1.1 ISOLATION OF BACTERIOPHAGES

Table 1 summarizes the origins of bacteriophages used in the project. Cultures of E. coli, Sh. dysenteriae, S. typhi and S. group D, grown on solid media containing 1.3% (w/v) nutrient agar and 2.5% (w/v) nutrient broth and inoculated with filtered liquid samples from the source as indicated in method 2.b.1 yielded ten bacteriophages. Three of these (SdRCL3, SdRCLI5 and SdRCL16) infected Sh. dysenteriae, four (StRCLI5, StRCL16, StRCL25 and SgRCL24) infected species of Salmonella and three (EcRCL24, EcRCL25 and EcRCL26) infected E. coli.

All the ten liquid samples collected from the sources at Tema and Legon, which were filtered and subsequently used to infect lawns of the appropriate indicator bacteria yielded plaques of different sizes. A typical example shows plaques of at least two different sizes produced on a lawn of bacteria (Fig. 2). Nine of the bacteriophages had similar morphology, although their tail lengths varied greatly. One bacteriophage possessed no tail. Typical examples of electron micrographs of bacteriophage are shown in Fig. 3. Several species of Shigella, Salmonella and Escherichia are apparently lytic for phages of these types (Koibong *et. al., 1961*). Figs. 3b and 3c however indicate that apparently there were two types of phages in purified isolates of each phage preparation.

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Phages SdRCL3, SdRCL16, StRCL16 and EcRCL24 have well defined and compact heads which were icosahedral in shape. Phage SdRCL15 possessed a head which was not well defined but was icosahedral (Fig. 3b). Phages EcRCL25 and EcRC16 had elongated heads which were icosahedral in symmetry (Fig. 3g and 3h). Phage StRCL15, StRCL25 and SgRCL24 possessed isometric heads (Figs 3d, 3i and 3j). The dimensions of phage heads obtained from micrograph measurements could not be adequately compared owing to some difference in magnifications, although it was observed that the heads were not of the same size.

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ORIGIN OF BACTERIOPHAGES

TABLE 1: Source of bacteriophages.

All the bacteriophages in the table below were obtained by infecting the indicator bacteria with filtered liquid samples from the source indicated.

BACTERIOPHAGES	INDICATOR BACTERIA	SOURCE
SdRCL3	*Sh. dysenteriae*	Sewage water, Legon Police Station Sewage Leakage
SdRCL15	"	Sewage water, Tema Sewage Treatment Plant (Lower Point)
SdRCL16	"	Sewage water, Tema Sewage Treatment Plant (Upper Point)
StRCL15	*S. typhi*	Sewage water, Tema Sewage Treatment Plant (Lower Point)
StRCL16	"	Sewage water, Tema Sewage Treatment Plant (Upper Point)
EcRCL24	*E. coli*	Sewage water, Tema General Hospital Sewage Leakage
EcRCL25	"	Gutter water, Tema main drainage into Sakumo Lagoon
EcRCL26	"	Sewage water, Legon main sewage Treatment Plant
StRCL25	*S. typhi*	Gutter water, Tema main drainage into Sakumo Lagoon
SgRCL24	*S. group D*	Sewage water, Tema General Hospital Sewage leakage.

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Samples designated Sd, St, Sg and Ec gave plaques when tested on *Sh. dysenteriae*, *S. typhi*, *S. group D* and *E. coli* respectively.

RCL designation of samples indicate that they were isolated by Rodrigues and Clement at Legon. A total of 26 samples were collected and the number of the sample test tube is the number of the bacteriophage.

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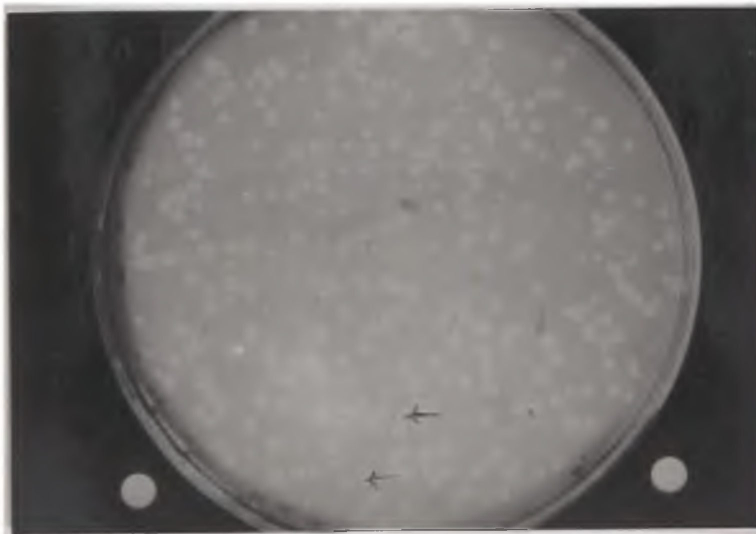

Fig. 2: Plaques produced on a lawn of indicator bacteria.

Plaques produced on a lawn of Sh. dysenteriae cells infected with phage SdRCL16.

There are different plaque sizes on the same lawn of indicator strain.

MAGNIFICATION X1

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Fig 3: Electron micrographs of negatively stained *Sh. dysenteriae*, *S. typhi*, *E. coli* and *S. group D* bacteriophages.

a. SdRCL3

b. SdRCL15

c. SdRCL16

d. StRCL15

e. StRCL16

f. EcRCL24

g. EcRCL25

h. EcRCL26

i. StRCL25

i. SgRCL24

Purified suspensions of bacteriophages were negatively stained with uranyl acetate (2%). The micrographs were observed under the HITACHI H600 transmission electron microscope.

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(i)

(ii)

(iii)

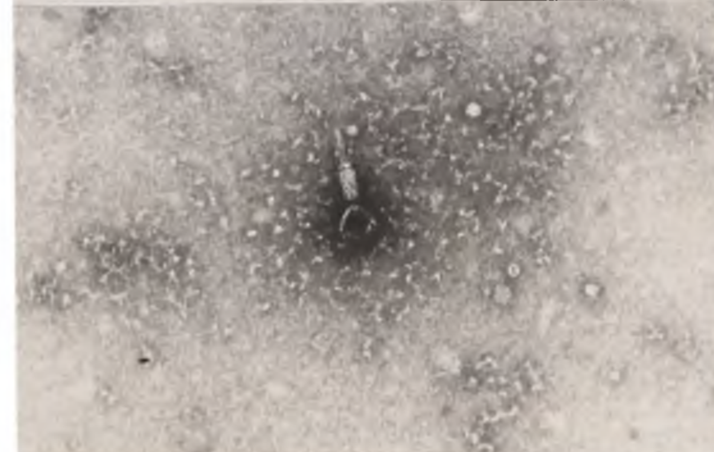

Fig. 3a: Electron micrographs of bacteriophage SdRCL3
MAGNIFICATION x 160,000.
(i) Icosahedral head and short tail.
(ii) Well defined tail sheath and base plate (knob)
(iii) Dense area of head contains the DNA.

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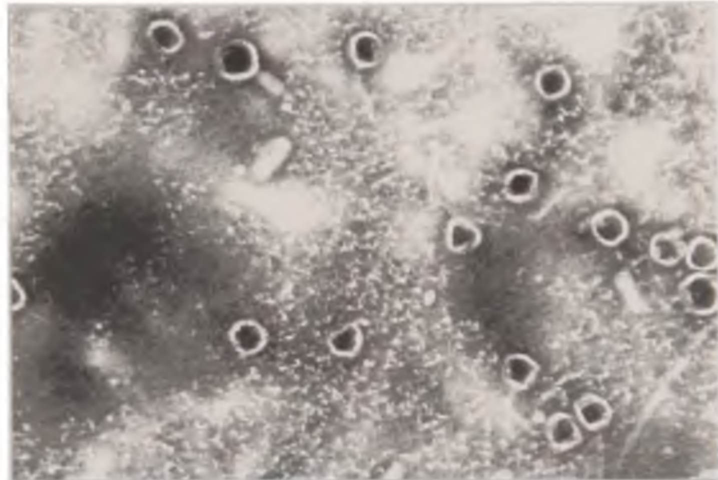

Fig. 3b: Electron micrograph of bacteriophage SdRCL15
MAGNIFICATION x 160,000.
Head undefined but icosahedral in shape.
Two types of phages, one with a short tail
covered by a sheath and the other without tail.

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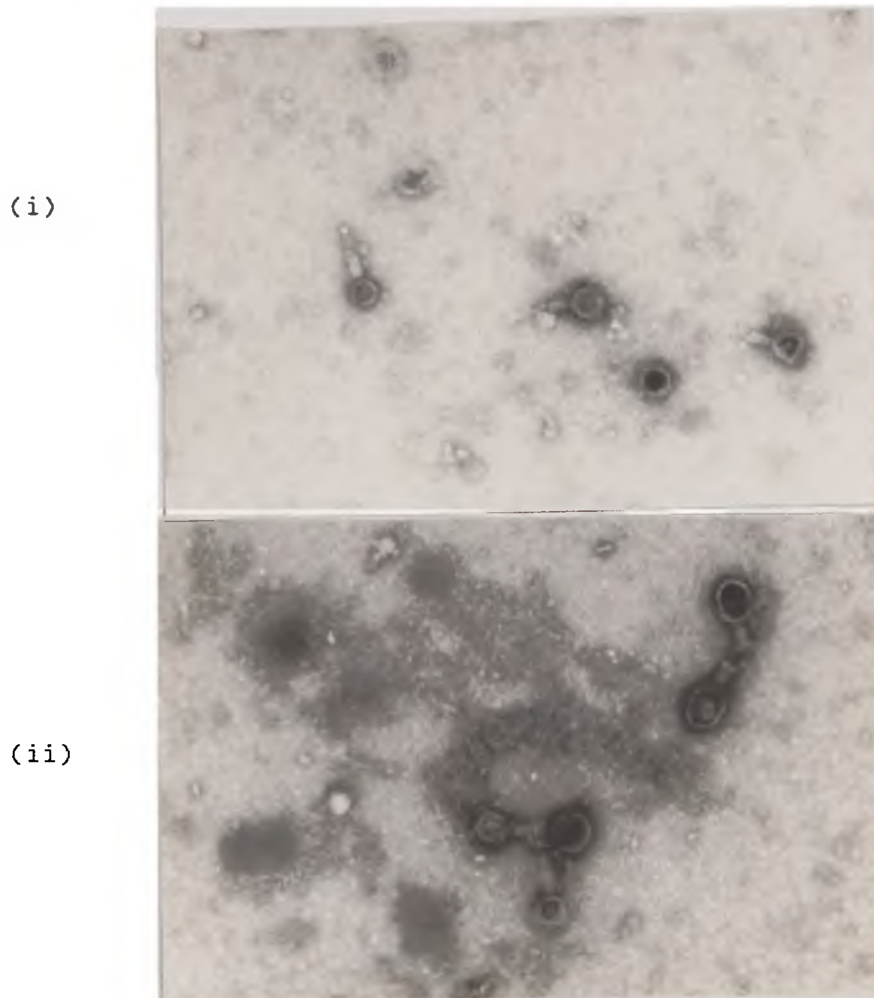

Fig. 3c: Electron micrographs of bacteriophage SdRCL16 MAGNIFICATION x 120,000.
(i) Icosahedral head and a long tail with sheath and knob.
(ii) Well defined compact head and contractile tail which looks short.

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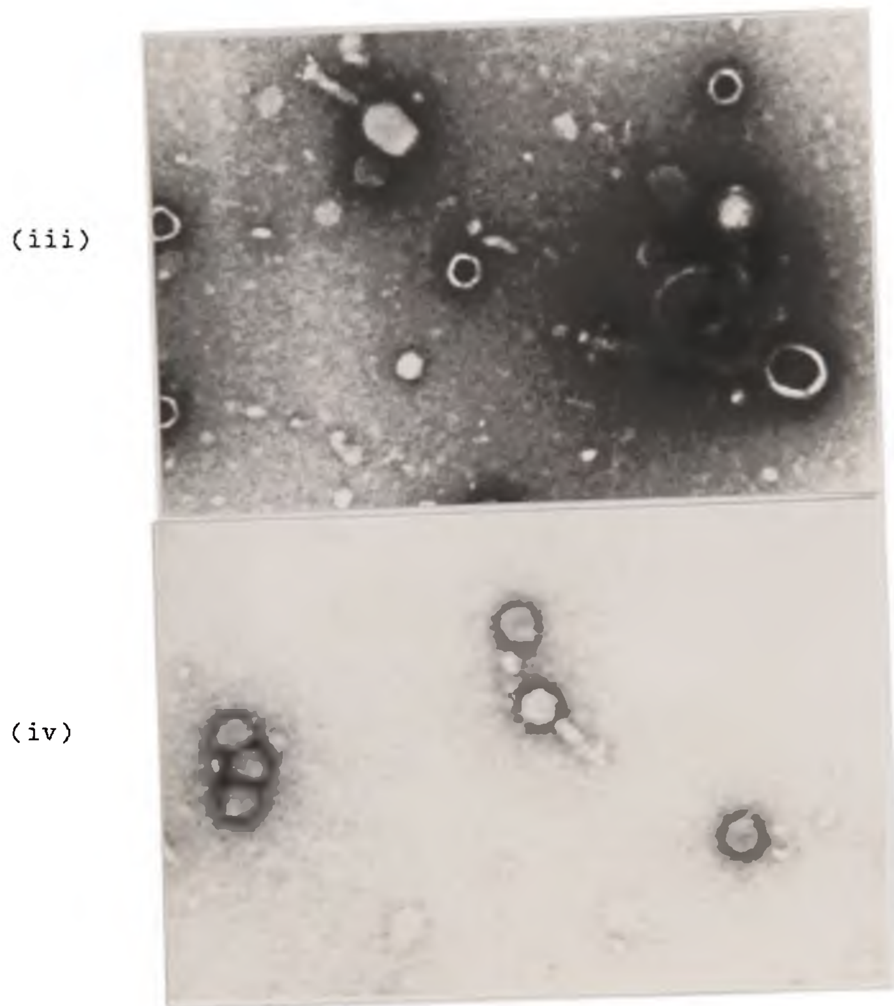

Fig. 3c: Electron micrographs of bacteriophage SdRCL16
MAGNIFICATION x 160,000.
(iii) Base plate which terminates in fibres.
(iv) Two types of phages one with tail and the other without tail.

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(i)

(ii)

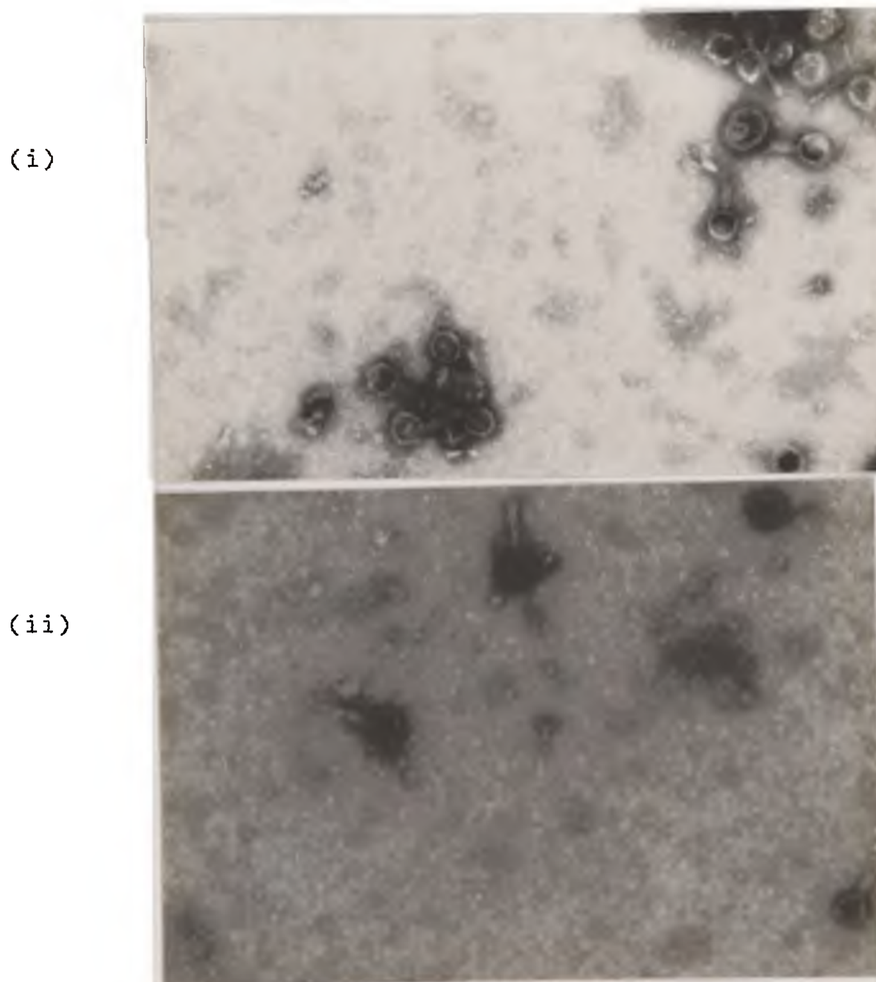

Fig. 3d: Electron micrographs of bacteriophages StRCL15
MAGNIFICATION x 120,000.
(i) Isometric head and a minimal tail.
(ii) Broken tail in medium.

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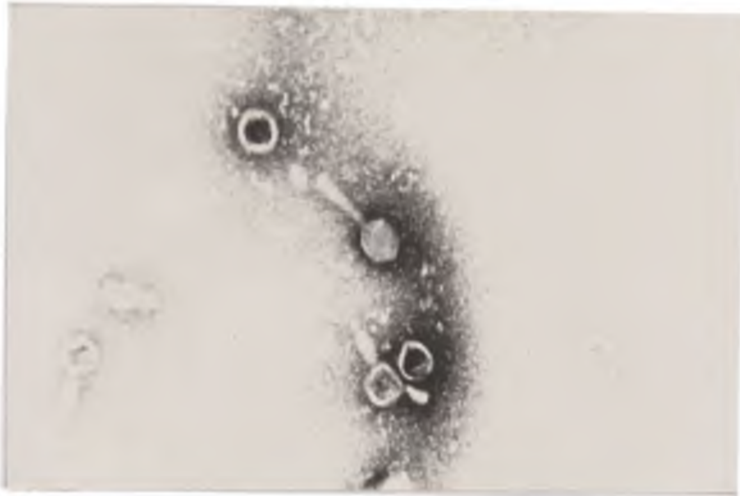
(i)



(i

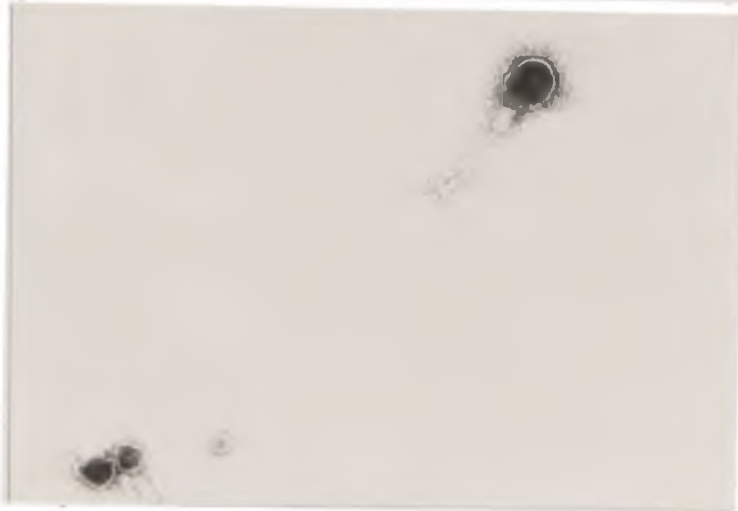


Fig. 3e: Electron micrographs of bacteriophage StRCL16
MAGNIFICATION x 160,000.
(i) A compact icosahedral head.
(ii) Long contractile tail bearing a sheath.

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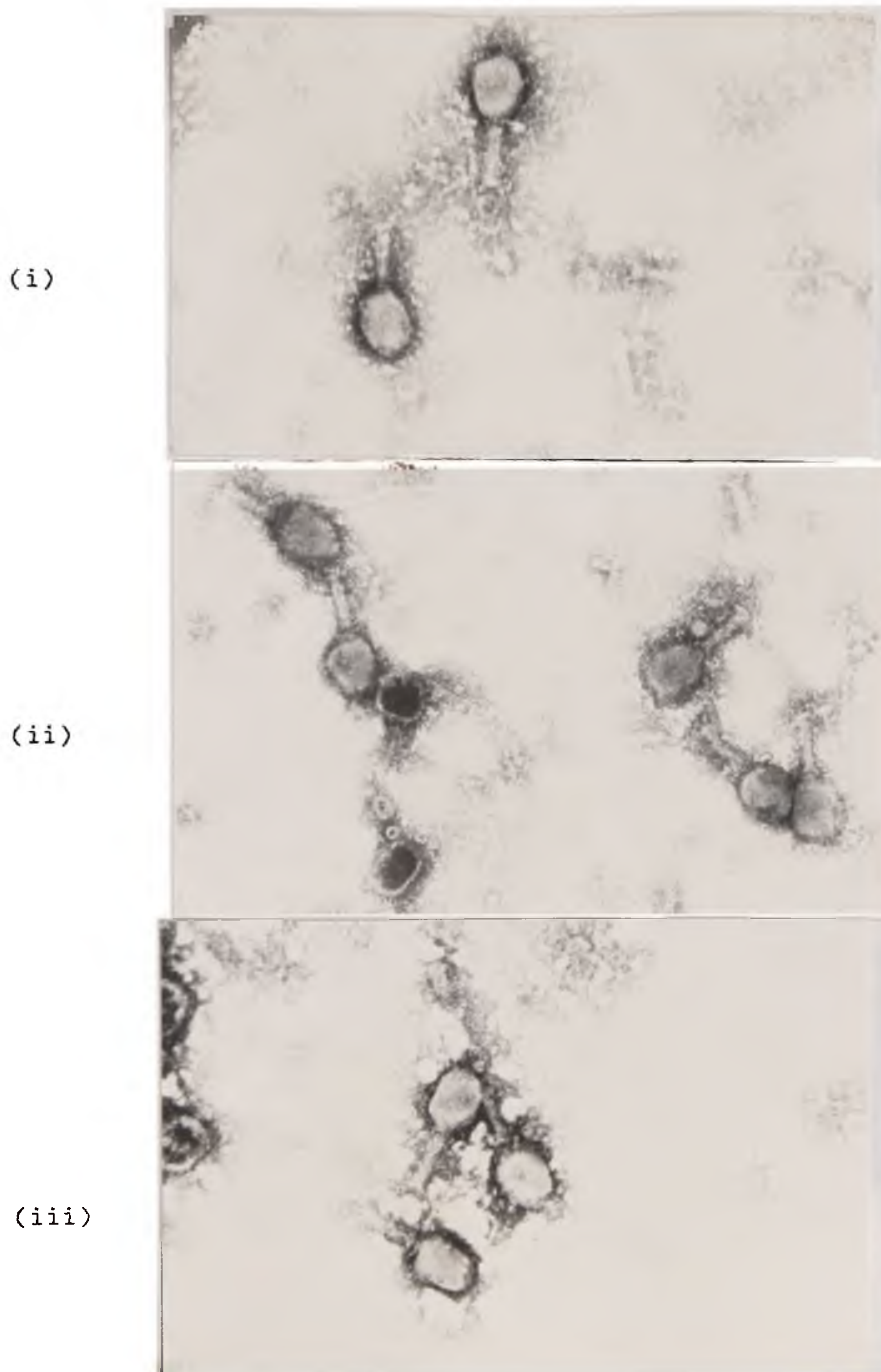

Fig. 3f: Electron micrographs of bacteriophage EcRCL24 MAGNIFICATION x 160,000.
(i) Icosahedral head and minimal tail.
(ii) Base plate as distal end of tail.
(iii) Colar at 'neck' of phage.

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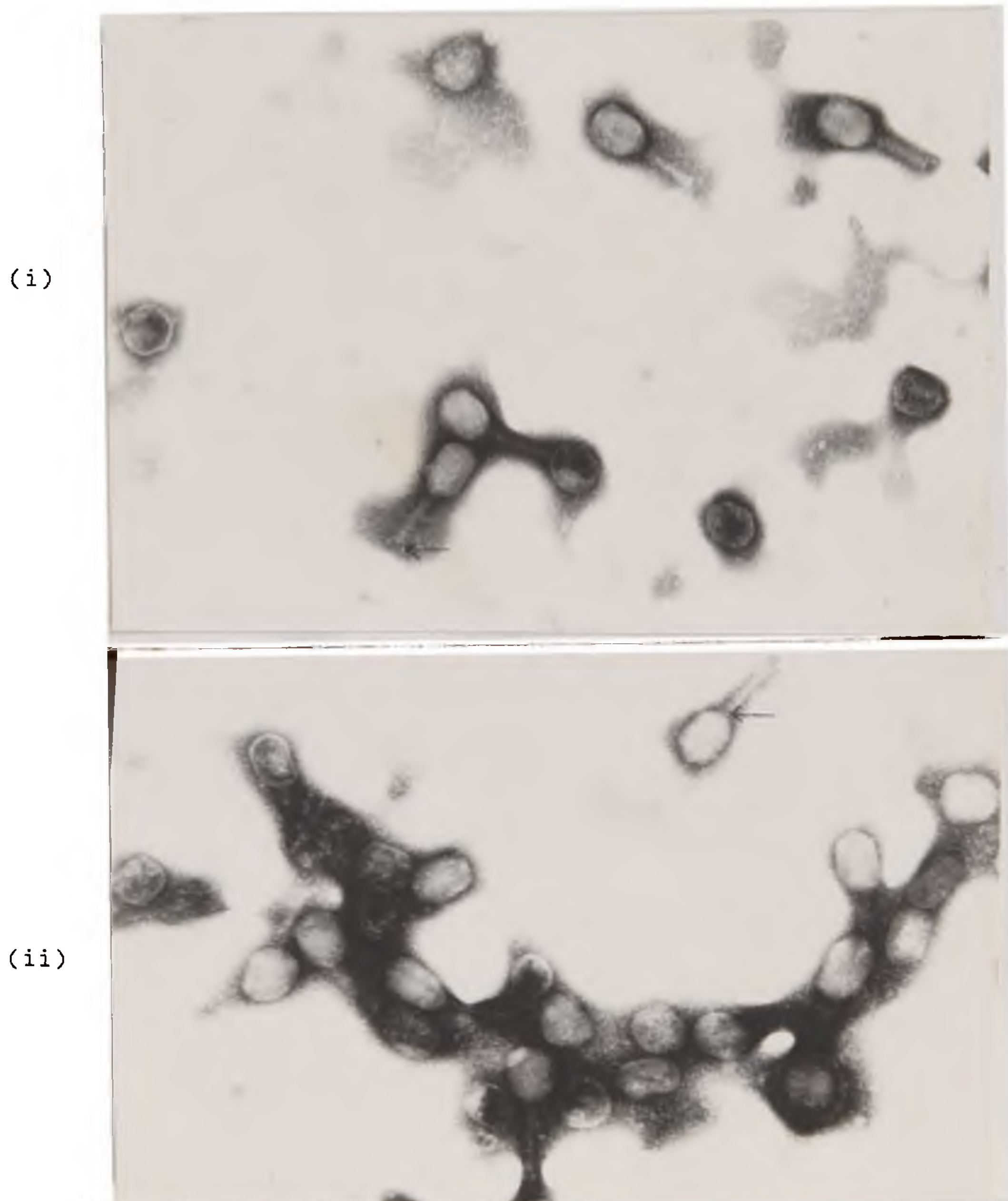
(i)



(ii)

Fig. 3g: Electron micrographs of bacteriophage EcRCL25 MAGNIFICATION x 160,000.
(i) Compact icosahedral head and minimal tail which terminates in a hexagonal base plate with tail fibres shown by arrow.
(ii) Colar clearly shown at 'neck' of phage shown by arrow.

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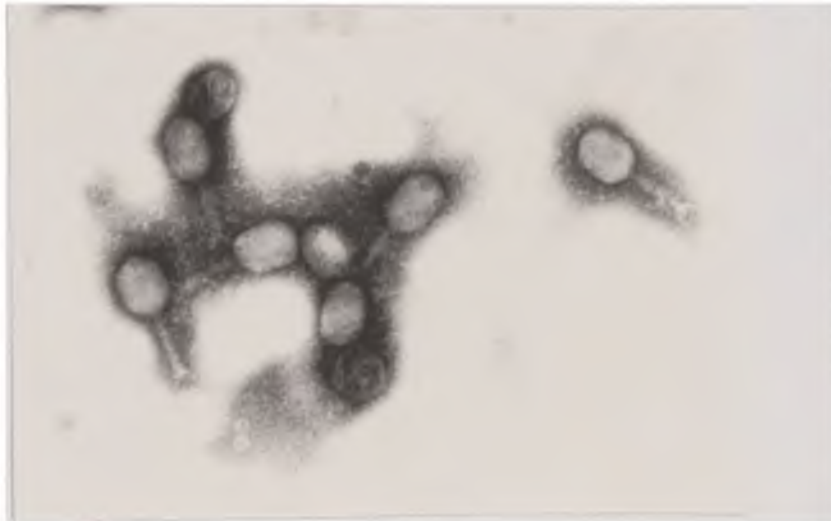

Fig. 3h: Electron micrographs of bacteriophage EcRCL26 MAGNIFICATION x 160,000.
Elongated icosahedral head and long tail without sheath, with knob and tail fibres.

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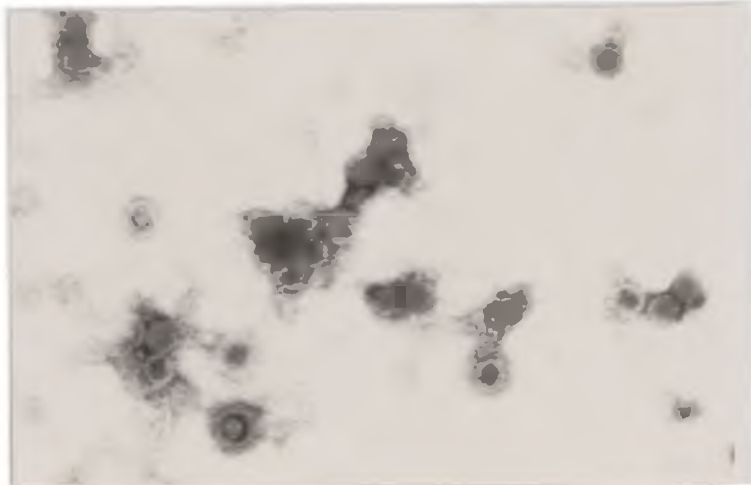

Fig. 3i: Electron micrograph of bacteriophage StRCL25
MAGNIFICATION x 160,000.
Phage with no tail.

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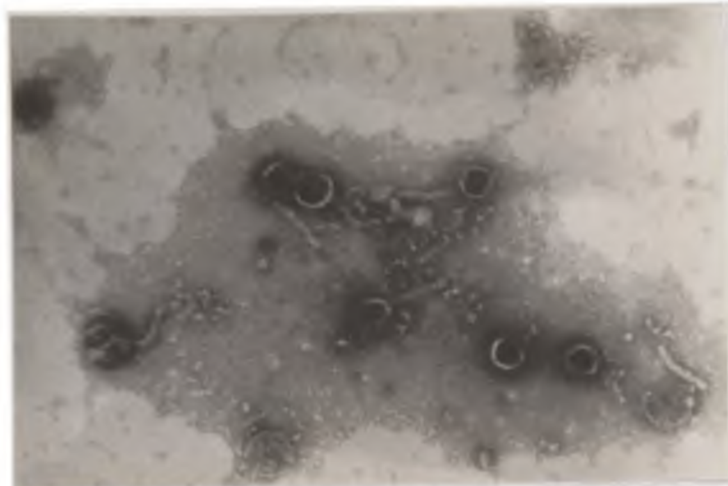

Fig. 3j: Electron micrograph of bacteriophage SgRCL24
MAGNIFICATION x 160,000.
Isometric head and long simple tail.

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TABLE 2: Bacteriophage typing of the ten isolated phages.

Summary of bacteriophage typing on phages for *Sh. dysenteriae*, *S. typhi*, *E. coli* and *S. group D*. (parameters of typing referred from Singleton, P. and Sainsbury, D. (1982) Dictionary of Microbiology and Molecular Biology 2nd Ed.)

BACTERIOPHAGE	PHAGE GROUP	VIRION MORPHOLOGY	HOST (INDICATOR)
SdRCL3	P1 (Family Myoviridae)	Icosahedral head (diameter 37.5nm) Short tail (length 62.5nm) complex covered by a sheath and bearing base plate.	*Shigella dysenteriae*
SdRCL15	P1 (Family Myoviridae)	Head not well defined but icosa-hedral in shape (diameter 43.8nm). Short tail (length 50.0nm) covered by a sheath.	*Shigella dysenteriae*
SdRCL16	P1 (Family Myoviridae)	Icosahedral head (diameter 40.63nm) long tail (length 93.8nm) complex, contractile, covered by a sheath, bearing a base plate (knob) and tail fibres.	*Shigella dysenteriae*
StRCL15	P22 (Family Myoviridae)	Isometric head (diameter 37.5nm). Minimal tail (length 58.3nm) covered by a sheath.	*Salmonella typhi*
StRCL16	P1 (Family Myoviridae)	Icosahedral head (diameter 43.7nm) long contractile tail (length 112.5nm) bearing a sheath.	*Salmonella typhi*

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TABLE 2: CONT'D

BACTERIOPHAGE	PHAGE GROUP	VIRION MORPHOLOGY	HOST (INDICATOR)
EcRCL24	T2 (T-even phage)	Icosahedral head (diameter 50.0nm) Minimal tail (length 75.0nm) without a sheath. The distal end of the tail terminates in a base plate.	*Escherichia coli*
EcRCL25	T4 (T-even phage)	Elongated head with icosahedral symmetry (dimensions 83 x 50nm) attached-via a neck to a tail (dimensions 100 x 16.7nm) with an outer contractile sheath. The distal end of the tail terminates in a hexagonal base plate which has tail fibres.	*Escherichia coli*
EcRCL26	T2 (T-even phage)	Elongated icosahedral head (68.8 x 50.0nm) long tail (dimensions 75.0 x 6.3nm) terminating in a base plate (knob).	*Escherichia coli*
StRCL25	PRD 1 (Family Tectiviridae	Isometric head (diameter 31.3nm) with internal lipid membrane. No tail.	*Salmonella typhi*
SgRCL24	P22 (Family Myoviridae	Isometric head (diameter 31.3nm) long simple tail (length 68.8nm) without a base plate.	*Salmonella group D*

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3.1. 2 SEROLOGICAL PREPARATION

Anti StRCL16 and anti EcRCL24 sera were raised in rabbits by intramuscular injection of the appropriate phage particles emulsified with complete Freund's adjuvant. Complete Freund's adjuvant contains heat-killed mycobacteria (Mycobacterium tuberculosis) that have the property of enhancing antigenicity in the animal by virtue of the endotoxin they possess; the adjuvant also helps as a carrier of the antigen into the phagocytic cells (Jones and Leskowitz, 1965).

The injections were carried out over such a period and in such amounts as to produce hyperimmunization to the animals; the animals were first screened to test the absence of such antibodies in their sera before the experiment was carried out. Phage samples of StRCL16 and EcRCL24 used had protein concentrations of 0.012mg/ml and 0.032mg/ml, determined by the method according to Lowry *et. al.,* (1951) (Fig. 4). The injections were first carried out with complete Freund's adjuvant and then subsequently using incomplete Freund's adjuvant. The sera were not titrated against phage proteins but they were assayed by determining the degree of inactivation of the homologous phages as well as the eight other phages by the method of Clowes and Hayes (1968), the rate of inactivation K is calculated from the relationship:

$$C = Coe^{-Kt}$$

where Co is the original phage concentration in p.f.u./ml and C the concentration after t minutes of incubation with antiserum. The value of the coefficient of inactivation, K for phage StRCL16 is 936 and phage EcRCL24 is 988 (Table 3).

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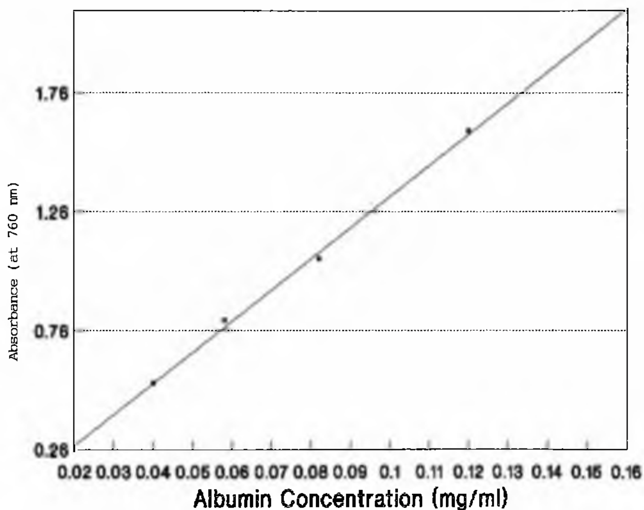

Figure 4: Calibration curve for the determination of protein concentration of antigenic materials.

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TABLE 3: Determination of K values of the ten isolated phages.

Coefficient of inactivation of bacteriophages by anti StRCL16 and EcRCL24 sera.

BACTERIOPHAGE	$K(min^{-1})$ Anti StRCL16	$K(min^{-1})$ Anti ECRCL24
SdRCL3	64	76
SdRCL15	59	84
SdRCL16	108	105
StRCL15	426	187
StRCL16	936	152
EcRCL24	126	988
EcRCL25	118	728
EcRCL26	132	594
StRCL25	54	196
StRCL24	49	13

The coefficient of inactivation of the bacteriophages (K) is calculated from the equation $C = Coe^{-Kt}$ (Clowes and Hayes, 1968).

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3.1. 3 DIGESTION OF PHAGE DNA WITH RESTRICTION ENDONUCLEASES HIND III AND Eco RI

Restriction endonucleases have proved very useful in dissecting genomes of a number of DNA viruses. Digestion of the DNA of the ten bacteriophages with the restriction endonucleases Hind III and Eco RI generated a series of linear duplex DNA fragments which were fractionated according to size on agarose gel (Fig. 5A and B). The pattern obtained thus represents the limit of digestion. As the distance migrated by each fragment on the gel is inversely related to the molecular weight for each of the fragments, an estimation of their sizes could be made directly (Table 4), using the molecular weights of fragments obtained by restricting lambda DNA with Hind III and Eco RI (Allet *et. al.*, 1973). Standard curves of log molecular weight versus electrophoretic mobility were constructed (Fig. 6), for the lambda DNA fragments and used for the estimation. The molecular weights of the fragments resolved on this gel system range from 0.125 to 33.113kbp. The fragment of molecular weight 23.000kbp was common to the ten phages with the exception of phage SgRCL24; also fragments 14.454 and 0.125kbp were common to phages 6 and 7. This observation compares favourably with the similarity of morphology of the phages obtained from electron microscopy (Fig. 3 c, d, e, f, g and h). Furthermore several of the fragments appeared as blotches, this was not due to overloaded gels since a decrease in the amount of digested DNA used did not show any change of pattern, but rather a decrease in the intensity of the bands (Fig. 5A and 5B).

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Fig. 5: Agarose gel electrophoresis of *Hind III* and *Eco RI* restriction endonuclease generated fragments of bacteriophage DNA.

A - *Hind III* digested lambda

A' - Undigested lambda

B - *Eco RI* digested lambda

B' - Undigested lambda

1 - SdRCL3

2 - SdRCL15

3 - SdRCL16

4 - StRCL15

5 - StRCL16

6 - RcRCL24

7 - EcRCL25

8 - EcRCL26

9 - StRCL25

10 - SgRCL24

Bacteriophage DNA was purified as described in section 2.b.5 and digested overnight with *Hind III* and *Eco RI* at 37°C Digestion was terminated by incubation at 70°C for 10 min. Approximately 0.4µg of cleaved DNA was loaded on the gel and the electrophoresis run at 50mA (100volts) for 1 hour at room temperature.

DNA bands were visualised under ultra-violet light, since the gel contained ethidium bromide (10mg/ml).

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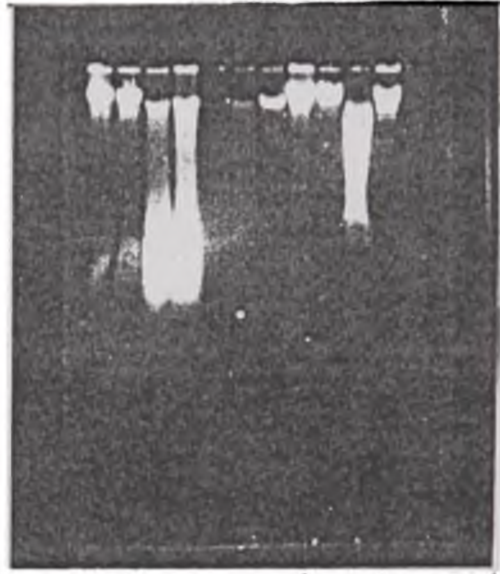
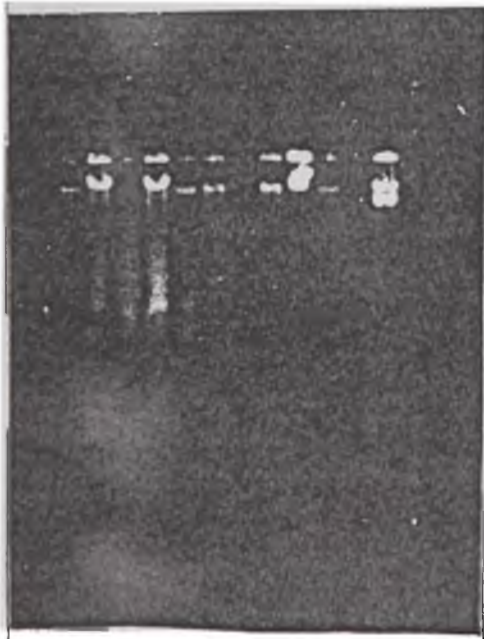
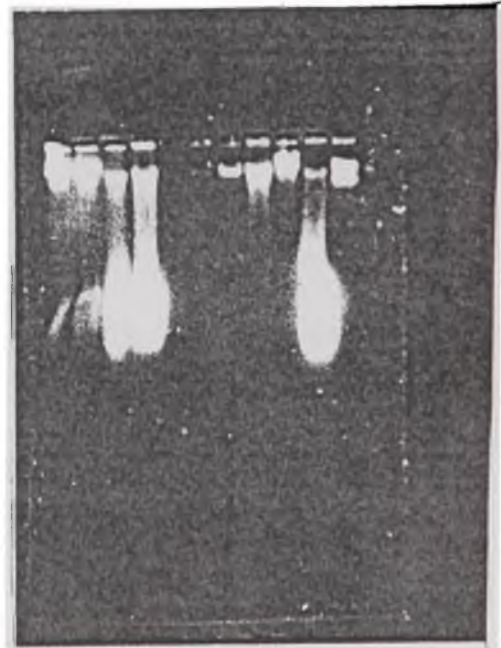
1',1,2',2,3',3,4'.4,5',5,A',A



6',6,7',7,8',8,9',9,10',10,A



1',1,2',2,3',3,4',4,5',5,B',B



6',6,7',7,8',8,9',9,10',10'B

Fig. 5: Agarose gel electrophoresis of restriction endonuclease generated fragments of the DNA of the ten isolated phages.

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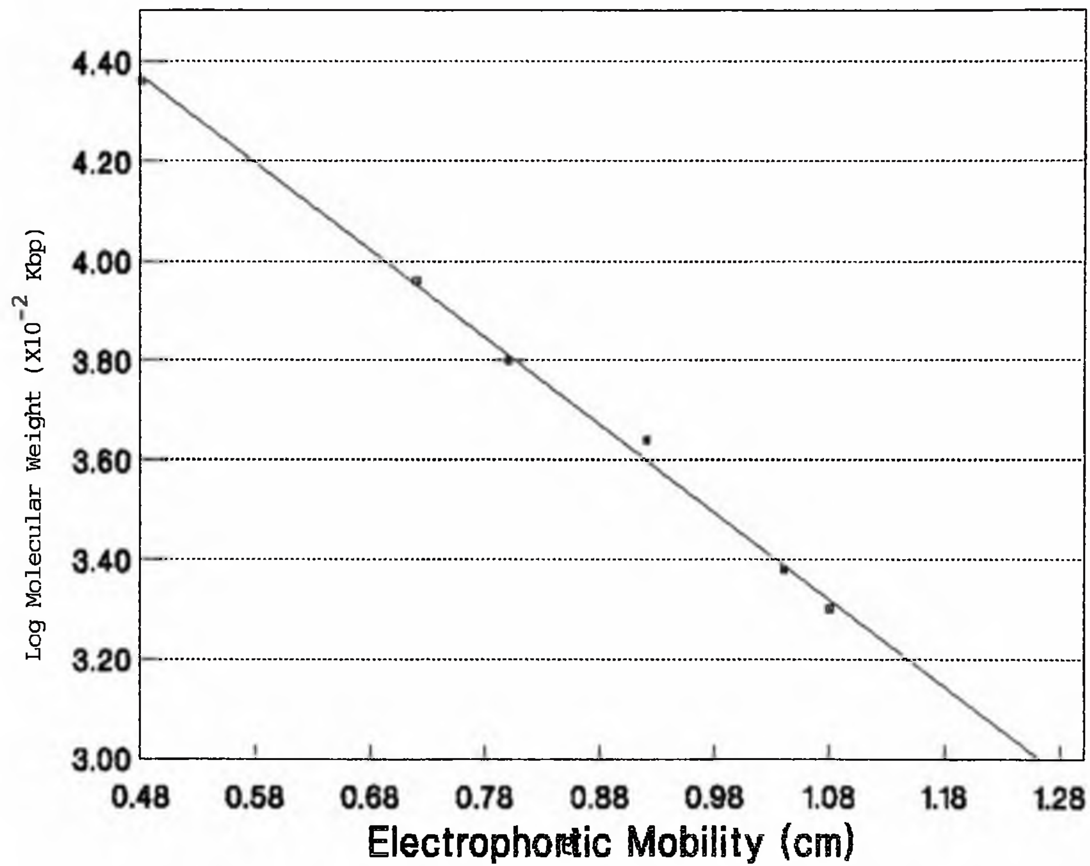

Figure 6: Calibration curve for the determination of the apparent molecular weights of the DNA fragments of the ten isolated phages.

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Fig. 6: Six *Hind III* endonuclease generated lambda DNA fragments were separated on 1.2 % (w/v) agarose gel. A straight line relationship is obtained when the logkbp of each of the fragment is plotted against electrophoretic mobility. From the direct proportionality relationship between logkbp and electrophoretic mobility the apparent molecular weight of DNA fragments generated were estimated (Fig. 5).

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TABLE 4: Molecular weight of the DNA fragments of the ten isolated phages generated by digestion of the phage DNA with Hind III and Eco RI (Fig. 5). The molecular weights of the fragments were determined from their electrophoretic mobilities (2.b. 12).

A - DNA fragments generated by Hind III

B - DNA fragments generated by Eco RI.

BACTERIOPHAGE		ELECTROPHORETIC MOBILITY (cm)	MOLECULAR WEIGHT OF DNA FRAGMENTS (kbp)
SdRCL3	A	0.5	23.000
	A	2.3	0.125
	B	0.4	33.113
SdRCL15	A	0.6	14.454
	B	0.5	23.000
	B	2.3	0.125
SdRCL16	A	0.6	14.454
	A	2.4	0.125
	B	0.5	23.000
StRCL15	A	0.6	14.454
	A	0.8	6.310
	B	0.5	23.000
StRCL16	A	0.4	33.113
	A	0.7	9.400
	A	1.8	0.125
	B	0.5	23.000
EcRCL24	A	0.5	23.000
	A	0.7	9.400
	A	2.7	0.125
	B	0.4	33.113
	B	0.6	14.454
	B	2.3	0.125

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TABLE 4: CONT'D

BACTERIOPHAGE		ELECTROPHORETIC MOBILITY (cm)	MOLECULAR WEIGHT OF DNA FRAGMENTS (kbp)
EcRCL25	A	0.5	23.000
	B	0.5	23.000
	B	2.2	0.125
EcRCL26	A	0.6	14.454
	B	0.5	23.000
StRCL25	A	0.5	23.000
	A	0.7	9.400
	A	1.8	0.125
	B	0.5	23.000
	B	0.7	9.400
SgRCL24	A	0.7	9.400
	A	2.2	0.125
	B	0.6	14.454
	B	2.1	0.125

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3.1. 4 SDS-PAGE OF SOLUBILIZED PHAGE PROTEINS

The apparent molecular weight range of the polypeptide bands was 11.7 to 199.5kd, with a preponderant number of the bands being either equal to or less than 73.3kd. Polypeptide band of apparent molecular weight of 12.9kd was present in six of the samples. Also bands that moved with a mobility of between 8.8 to 9.2 cm and of molecular weight range 12.9 to 14.8kd were common to all the ten phage samples. A polypeptide band of apparent molecular weight 3.8kd was common to phages SdRCL15 and SdRCL16. Polypeptide band of apparent molecular weight 15.5kd was present in all phage samples active on *S. typhi*. Also band 12.9kd was common to all the phages active on *E. coli*. Phages EcRCL25 and EcRCL26 were unique for possessing a polypeptide band of apparent molecular weight of 199.5kd.

The yield of polypeptide bands was not entirely dependent on the indicator bacteria strain, on which the phage was active. Phages active on *Sh. dysenteriae* possessed a few polypeptide bands. Generally phages active on *S. typhi*, *E. coli* and *S. group D* yielded more polypeptide bands, with the exception of phages StRCL15, StRCL16 and EcRCL24 (Fig. 5) Eight of the phages were from the Tema Sewage Treatment plant whilst only two namely SdRCL3 and EcRCL26 were from the Legon vicinity

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Fig. 7: Four molecular weight marker proteins were separated on SDS-PAGE of 10% gel. A straight line relationship is obtained when the log. molecular weight is plotted against electrophoretic mobility (Fig. 7a). From the direct proportionality relationship between log. molecular weight and electrophoretic mobility the apparent molecular weights of phage proteins of the ten bacteriophages were estimated (Fig. 7b).

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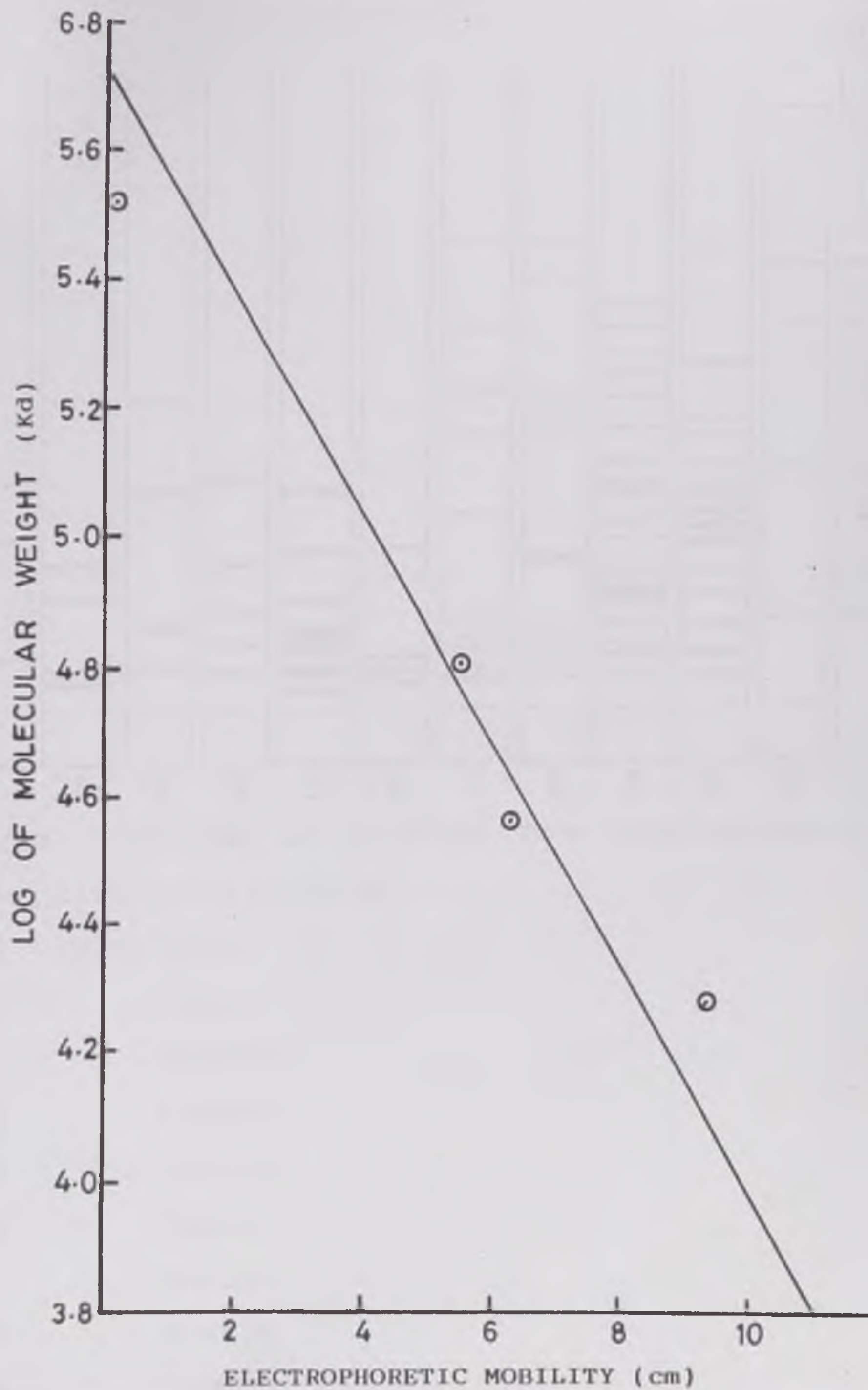

Fig. 7a: Calibration curve for the determination of apparent molecular weights of the proteins of the ten isolated phages.

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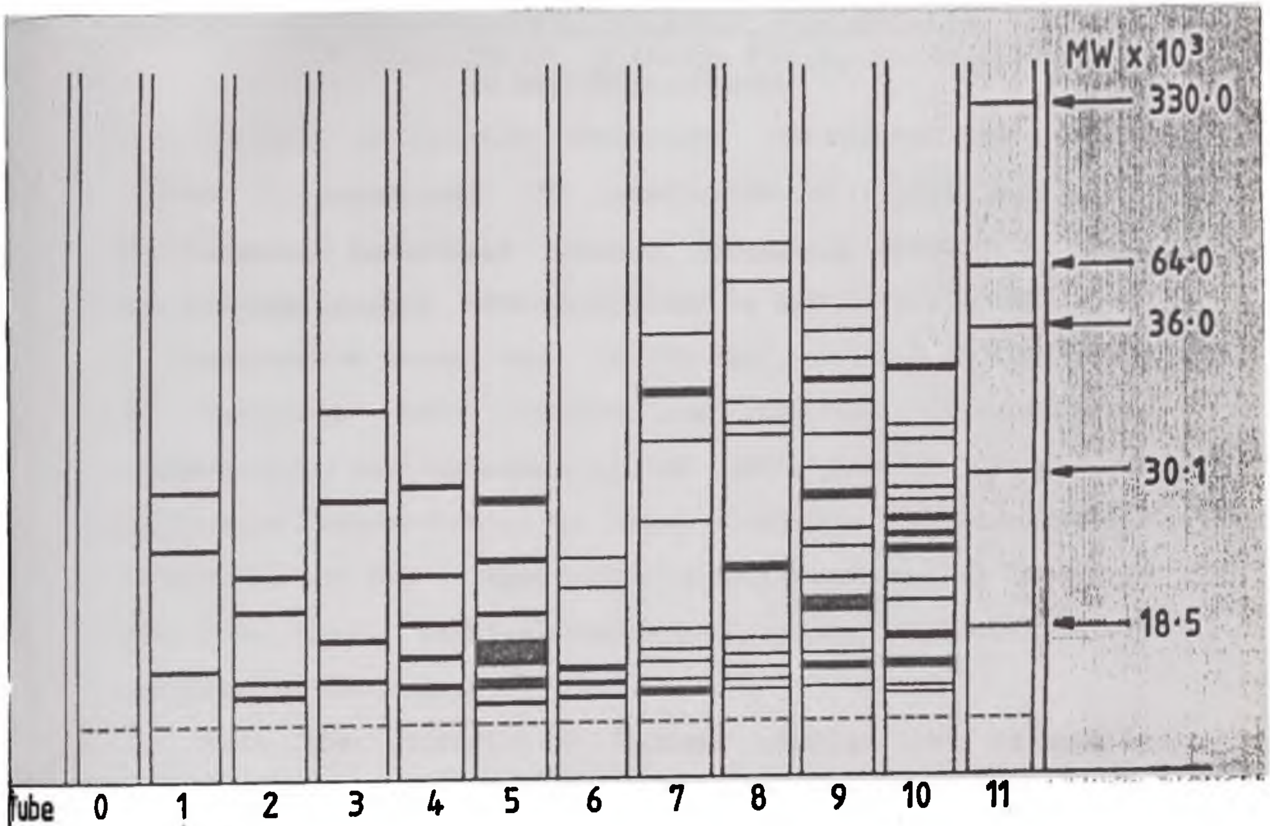

Fig. 7b: SDS-PAGE of proteins from purified phages run together with marker proteins.

TUBE 1 - PHAGE SdRCL3

" 2 - " SdRCL15

" 3 - " SdRCL16

" 4 - " StRCL15

" 5 - " StRCL16

" 6 - " EcRCL24

" 7 - " EcRCL25

" 8 - " EcRCL26

" 9 - " StRCL25

" 10 - " SgRCL24

" 11 - MOLECULAR WEIGHT MARKER PROTEINS

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3.1.5 MINIMUM ANTIBIOTIC INHIBITORY CONCENTRATION (MIC) AND INDUCTION OF INCREASED ANTIBIOTIC TOLERANCE IN BACTERIAL STRAINS

Minimum antibiotic inhibitory concentrations (MICs) against Ps. aeruginosa, Sh. dysenteriae, S. aureus, S. typhi, Sh. flexneri, Salmonella group D, Klebsiella species, E. coli and Proteus species were determined by agar dilution which is a quantitative method that allows the counting of the number of bacterial cell colonies in relation to antibiotic concentration and consequently the estimation of the minimum antibiotic concentration at which there is bacterial growth inhibition or MIC. Agar dilution therefore has an advantage over the routine clinical sensitivity tests, which are purely qualitative techniques.

With the exception of Proteus species and Salmonella group D which are sensitive at 5µg/ml concentration of tetracycline while S. typhi is sensitive at 10µg/ml, the MICs for the various bacterial strains estimated indicate an increased level of tolerance for the various antibiotics (Fig. 8)

Antibiotic resistance bacterial strains were induced by the training method (see section 2.b.16). It is commonly found that a decrease in sensitivity to antibiotic is gradual, so that the concentration of the drug can only be raised by small steps at each successive subculture. Bacterial strains, like S. typhi and Sh. flexneri. showed an exceptional increased tolerance for penicillin-V-sulfoxide and erythromycin, whilst Salmonella group D. Sh. flexneri. E. coli and Proteus species showed similar results with

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penicillin-V-sulfoxide and erythromycin.

Generally, the pattern of MIC for each of the bacterial strains varied with the different antibiotics and no two strains showed a definite relationship; however it was observed that all the strains showed an increased tolerance for penicillin-V-sulfoxide.

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Fig. 8: Eleven bacterial strains were used in the MIC studies and increased tolerance level determinations made by the agar dilution method.

The MICs are the antibiotic concentrations with no bacterial cell growth, and indicated on the graphs by the points where the curves terminated.

The figures in brackets indicated against the strains of bacteria show MIC values and the increased tolerance levels respectively of the antibiotic for each bacterial strain.

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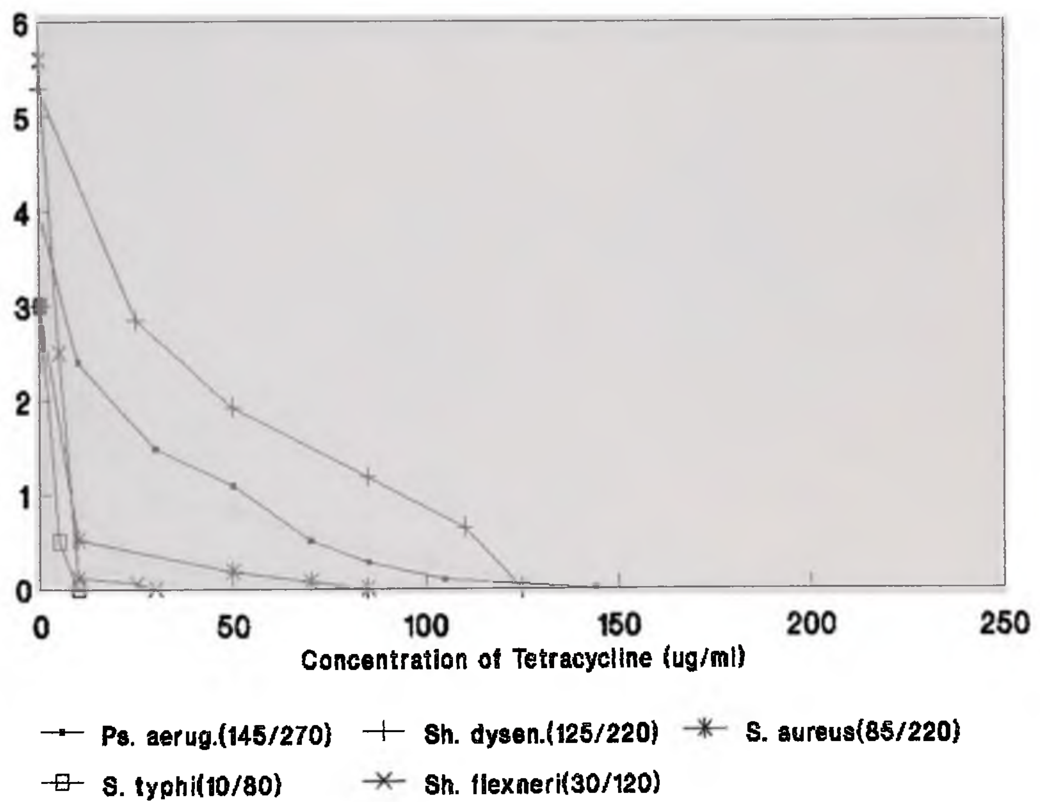

Fig. 8a: Determination of the MICs for tetracycline

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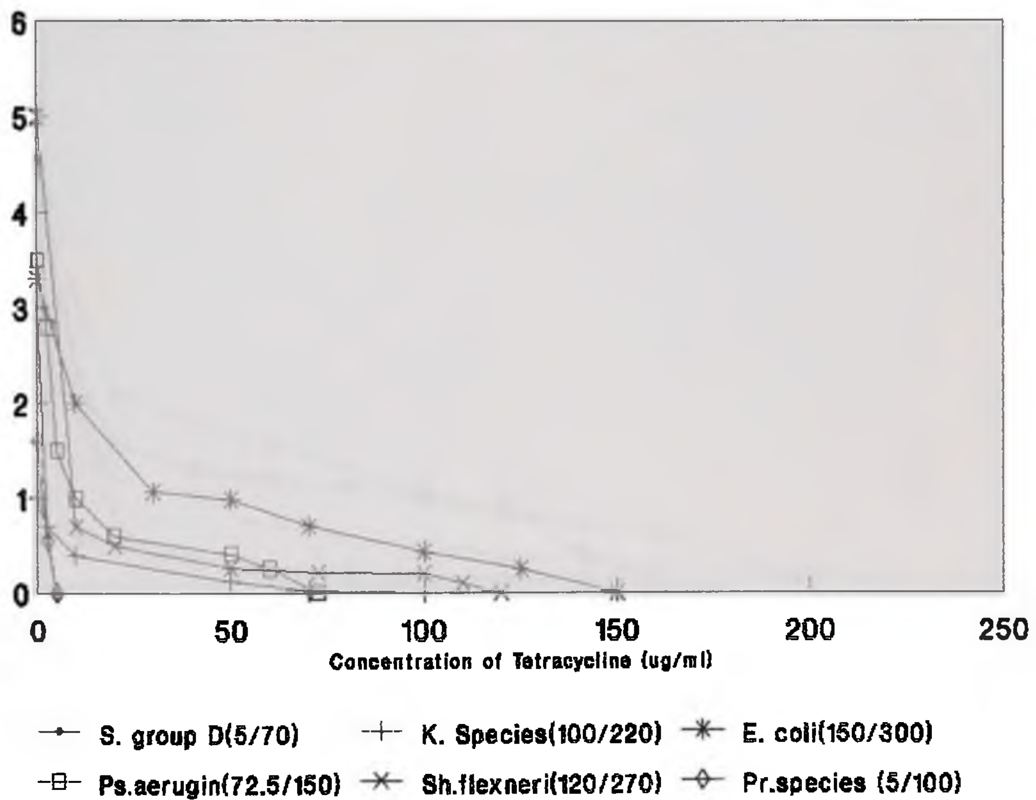

Fig. 8b: Determination of the MICs for tetracycline

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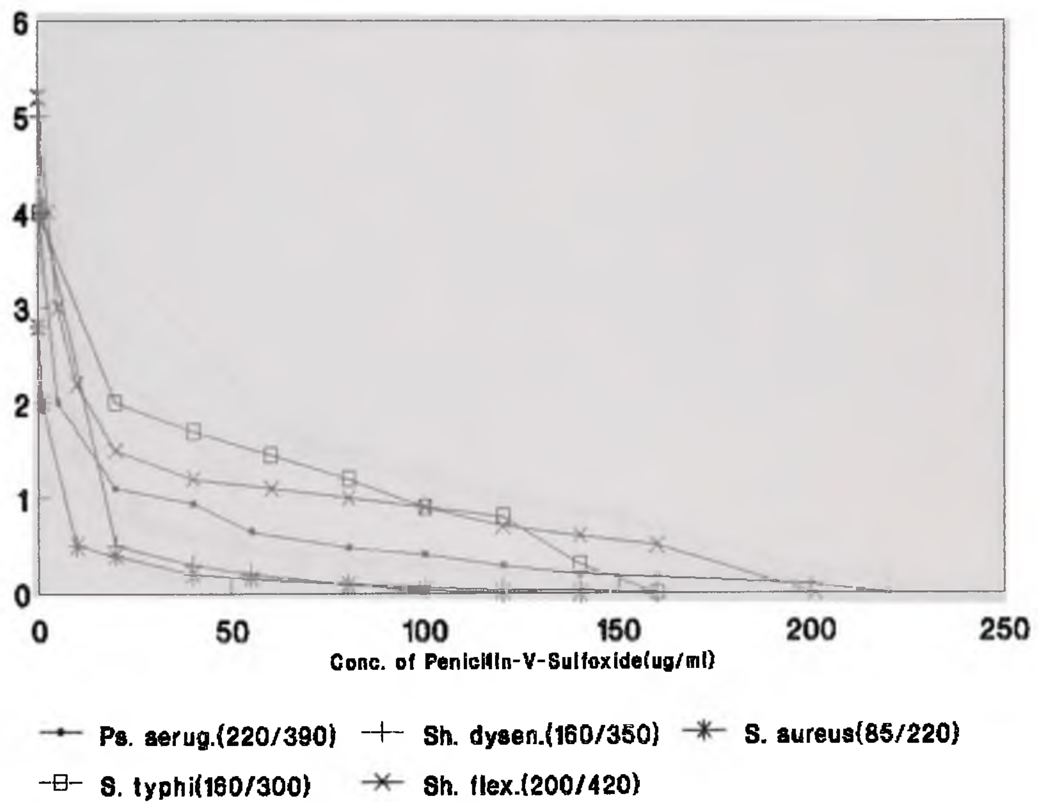

Fig. 8c: Determination of the MICs for Penicillin-V-sulfoxide

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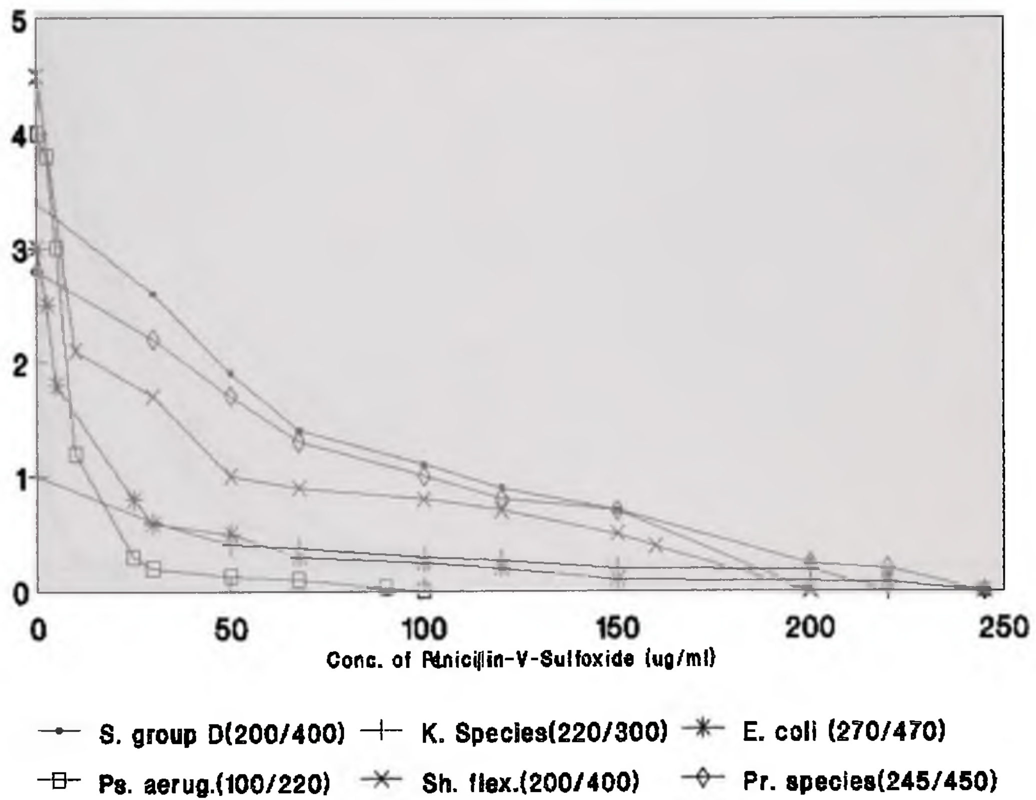

Fig. 8d: Determination of the MICs for Penicillin-V-sulfoxide

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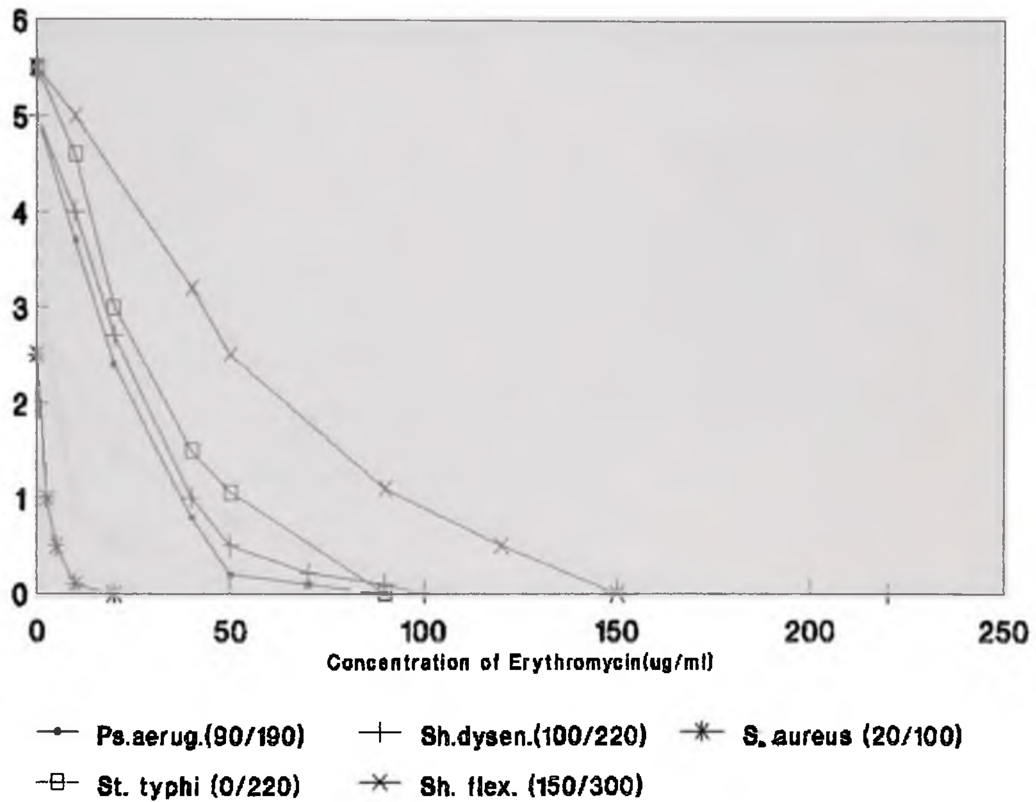

Fig. 8e: Determination of the MICs for erythromycin

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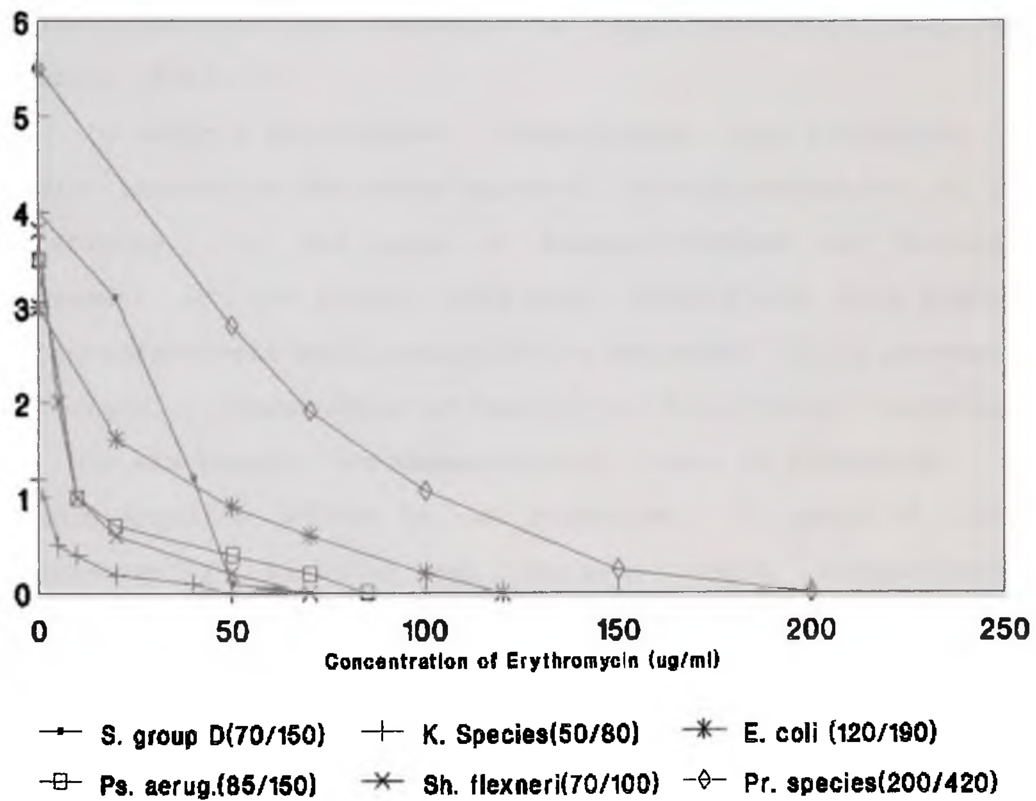

Fig. 8f: Determination of the MICs for erythromycin

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3.1. 6 TRANSDUCTION

Some of the phages were screened for their ability to transduce three markers: resistance to tetracycline, penicillin-V-sulfoxide and erythromycin among selected bacterial strains.

Six bacteriophages, namely SdRCL3, SdRCL15, StRCL15, StRCL16, EcRCL24 and EcRCL25 were found to be capable of transducing all the markers in their indicator bacterial strains (Table 5).

In control experiments, these phages were propagated on their respective indicator bacteria strains sensitive to the antibiotic. In the case of phages StRCL16 and EcRCL24, treatment of the phages with anti StRCL16 and anti EcRCL24 sera respectively before addition to recipient cells prevented infection. Transduction of penicillin-V-sulfoxide resistance by the six phages was comparatively lower in frequency. An allele specific effect is not suspected. A total of three independently isolated pen mutants, three independently isolated tet mutants and three independently isolated ery mutants were tested as donors for transduction mediated by one of the six phages, and in every case the transduction frequencies for tet and ery were between two to three times lower than pen (Table 5).

Liquid cultures of all transductant colonies did not release phage during growth. The transductants maintained their tet^R, pen^R and ery^R phenotype after several generations of growth in medium lacking the antibiotic. When used as indicator cells to phage that led to their transduction,

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transductants supported plaque multiplication with 100% efficiency These findings suggest generalized transducing phages.

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TABLE 5: Transduction frequencies for three tetracycline resistance alleles (tet^{-R}), three penicillin-V-sulfoxide resistance alleles (pen^{-R}) and three erythromycin resistance alleles (ery^{-R}) by six specific transducing phages.

Transduction frequencies ($X10^{-8}$ transductants/p.f.u.)

BACTERIOPHAGES

A.

DONOR BACTERIA STRAIN	SdRCL3	SdRCL15
Sh. dysenteriae tet^{-1}	0.86	0.45
Sh. dysenteriae pen^{-1}	1.83	2.74
Sh. dysenteriae ery^{-1}	0.66	0.46

B.

DONOR BACTERIA STRAIN	StRCL15	StRCL16
S. typhi tet^{-2}	1.25	0.30
S. typhi pen^{-2}	3.03	3.89
S. typhi ery^{-2}	0.98	0.72

C.

DONOR BACTERIA STRAIN	EcRCL24	EcRCL25
E. coli tet^{-3}	0.56	0.92
E. coli pen^{-3}	3.96	3.12
E. coli ery^{-3}	0.98	0.35

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CHAPTER 4

4.1 DISCUSSION AND CONCLUSION

4.1. 1 DISCUSSION

The alternative method of Yamamoto *et. al.,* (1970) was employed for the purification of the phage particles from which DNA was extracted. This simpler scheme yields phage preparations that are not as pure as those obtained from the more widely used density gradient centrifugation in sucrose and in caesium chloride for the purification and separation of particles with differences in nucleic acid content and composition (Cohen, 1960). However the electron micrographs (Fig. 3) show that the phage preparations are clean enough for subsequent characterization based on morphology, antigenicity, restriction fragment lengths of the DNA and protein profile.

Small viruses are built up by regular packing of identical protein subunits (capsomeres) resulting in two types of symmetry; a cylindrical shell having a helical (screw) symmetry, and a spherical shell which may be icosahedral. Electron micrographs showed that phages SdRCL3, SdRCL15, SdRCL16, StRCL15, StRCL16, and SgRCL24 resembled P phages while EcRCL24, EcRCL25, and EcRCL26 were like T-even phages (Fig. 3a, 3b, 3c, 3d, 3e, 3f, 3g, 3h, and 3j). The heads of most of the phages were icosahedral in symmetry (Fig. 3a, 3b, 3c, 3e, 3f, 3g, and 3h).

The tail is attached to the capsid on an axis of sixfold symmetry. The tail core may be hollow and surrounded by a contractible sheath. Some phages have sheathless tails which

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terminate in a 'knoblike' structure or base plate. Only phage StRCL25 had no tail (Fig. 3i) and it is a known fact that not all phages possess tails. However observation of the micrographs suggest more than one type of phage some without tails in especially phages StRCL15 and EcRCL24 (3d and 3f). A possible explanation for phages without tails may be from mechanical agitation, which may have caused the severing of the head from the tail during the purification. Phage typing indicated that phages SdRCL16 and StRCL16 have long contractile tails, phage EcRCL25 has a tail with an outer contractile sheath and some phages have either short, minimal or long simple tails (Table 2). Furthermore, some phages, notably SdRCL3, SdRCL16 and EcRCL25 (Fig. 5 3a, 3c and 3g), were structurally more sophisticated in that they possessed tail sheaths, base plate (knob) and tail fibres, indicative of the fact that the level of phage complexity is on the basis of their tail structures.

An important observation made during the isolation and propagation of the phages was the difference in plaque sizes on the indicator bacteria strain, for example, SdRCL16 grown by confluent lysis (Fig. 2). An explanation is that some of the phages notably SdRCL16, StRCL16 and EcRCL25 were clearly of more than one morphology which may probably account for the difference in plaque size. Bacteriophages are host specific, meaning phage, active on Sh. dysenteriae will not, for example, attack E. coli.

Electron micrographs showed that phage, active on Sh. dysenteriae had morphologies somewhat similar to those active

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on *S. typhi*, but the former would not attack the indicator bacteria strain of the latter and vice versa. The phage tail will recognize and adsorb to specific receptor sites on the cell wall of the appropriate bacterium. These sites occur within a particular chemical configuration into which the tail protein fits, similar to how a substrate or co-enzyme attaches itself to an enzyme (Oginsky and Umbreit, 1959). Thus the activity of a phage on bacterial host cells is not based on differences or similarity in morphologies but rather on differences in the recognition factor of the tails. This may be attributed to the tail proteins which will recognize a particular receptor site on the bacterial cell wall, hence if morphologically different but the same with respect to tail protein, they will attack the same bacterial host cells. In addition, a bacterial cell may possess more than one type of receptor site on its surface, making it susceptible to attack by more than one phage type.

The molecular weight of restriction fragment lengths of the DNA resolved on the agarose gel system used ranged from 0.125 to 33.113kbp. The fragment of molecular weight 23.000kbp was common to nine phages, the only exception being phage SgRCL24 [Fig. 5 (i) and (ii)], also fragments 14.454 and 0.125kbp were common to six and eight phages respectively. Since fragment 23.000kbp was common to nine of the phages and generally the phages had similar morphologies, this fragment may influence phage morphology. Secondly, restriction fragment length polymorphs of 0.125 and 23.000kbp indicate that phages SdRCL13 and SdRCL15 are closely related (Fig. 5A

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and 5B). Also the blotches that appeared on the electrophoresis were DNA fragments with molecular weights very close to one another and the enzymes used Hind III and Eco RI had several restriction sites on the DNA of the bacteriophages. The blotches were therefore several DNA fragments lying very close to one another and appearing as continuous bands.

SDS-PAGE has been a reliable method for obtaining separation of proteins and other biological macromolecules. Thus polypeptide chain sizes of phage capsids can be determined by comparing their electrophoretic mobilities to those of marker proteins. The protein profiles obtained by SDS-PAGE showed that the number of polypeptide bands varied widely, from three for SdRCL3 to sixteen for StRCL25 (Fig. 7b). This variation can be explained on the basis of the fact that phages EcRCL25, EcRCL26, StRCL25 and SgRCL24 that had many bands, definitely had a lot more DNA that coded for the synthesis of proteins of different sizes. Also the small amount of nucleic acid present in the virus can only code for a few kinds of amino acid residues, thus in viruses, protein coats are made up of a large number of one protein subunit, as present in phage StRCL16 or a few kinds of protein subunits, typified by phages SdRCL3 and SdRCL15 Fig. 7b). However, there is an exception to this general rule; phage T4, to which some of these phages resemble morphologically, is known to contain more than twenty unique protein bands.

Comparison of the polypeptide bands of phage is more appropriate when molecular weight ranges, are considered.

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Since conditions differ in the tubes like slight differences in the dimensions, uneven circulation of ions from the buffer and flaws in the setting of the gel, it is possible for bands of the same molecular weight to have small differences in mobilities. The apparent molecular weight range of the polypeptide bands of the ten phage samples was from 11.7 to 199.5kd, however most of the bands were either equal to or lower than 73.3kd (Fig. 7b). For the T4 phage, virion proteins have been found to range from 10.0 to 150.0kd (Neurath and Hill, 1975). The similarity is evident between these polypeptide bands and the proteins responsible for the morphology of phage T4. Also bands of apparent molecular weight range 12.9 to 14.8kd were common to all the ten phage samples (Fig. 7b). It is possible that this molecular weight range may actually be one polypeptide or closely related polypeptides which correspond to a protein essential in phage structure.

Polypeptide band of apparent molecular weight 13.8kd was common to phages SdRCL15 and SdRCL16. A band of apparent molecular weight 15.5kd was present in all phage samples active on S. typhi (Fig. 7b). Also band 12.9kd was common to all phages active on E. coli. Secondly, phage EcRCL25 and EcRCL26 were unique for possessing a polypeptide band of apparent molecular weight of 199.5kd that was higher than those of all the other phage samples. This could be due to proteins peculiar to these phages, which may play specific roles, such as proteins in the phage tails which recognize a receptor site on the host bacterial cell surface, or could be

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proteins in the tail that act as enzymes which lyse the peptidoglycan layer of the host bacterial cell wall during infection.

Phages serve as good antigenic material, capable of stimulating antibody formation when introduced into an animal. The introduction was done by intramuscular injection which leads to a much higher antibody titre because of the relatively slower absorption of the antigen by the animal (Haurowitz, 1968). Since the outer coat of the virus particle is comprised of proteins, it is reasonable to assume that the antibodies produced against the virus will complex with viral surface proteins (Hershey and Chase, 1952; Lanni and Lanni 1953). The value of the coefficient of inactivation, K, for phage StRCL16 is 936 and EcRCL24 is 988; the corresponding K values for the other EcRCL phages were substantially high and indicate an appreciable cross reactivity. The EcRCL phages are therefore closely related in DNA composition and protein structure (Table 3).

Genes are transferred among bacteria in one direction, from donor to recipient. In most cases, only part of the DNA is transferred and may align with the corresponding segment on the recipient's existing chromosome. The DNA may then recombine by breakage of the host chromosome and reunion of the free ends with the newly received DNA fragment that becomes stably incorporated into the recipient's chromosome. The excised fragment is destroyed by the enzyme DNase.

Although the two DNA segments are homologous, the recipient cell may receive a different form of a gene, thereby

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acquiring a new property from the donor cell. For example, a mutant, nonfunctional gene may be replaced by a homologous wild-type gene from the donor. If the altered characteristic or trait is observable, then it is indicative that genetic transfer has occurred, which may have a profound impact on the outcome of infectious disease in humans.

The three mechanisms for gene transfer in bacteria are transformation, transduction and conjugation. Some of the phages, namely SdRCL3, SdRCL15, StRCL15, StRCL16, EcRCL24 and EcRCL25 were screened for their ability to transduce three markers; resistance to tetracycline, penicillin-V-sulfoxide and erythromycin, and were found to be capable of transducing all the markers in their indicator strains of Sh. dysenteriae, S. typhi and E. coli.

It was found that the transductants maintained their antibiotic resistance phenotype after several generations of growth in medium lacking the antibiotic; also when used as indicator cells to phages that led to their transduction, transductants supported plaque multiplication with 100% efficiency. These findings suggest generalized transducing phages. This compares with transduction by E. coli phage P1 which is known to be the result of a small fraction of particles in a lysate containing bacterial, instead of phage DNA (Ikeda and Tomizawa, 1968).

The average transduction frequencies of the three markers tested are about 3 x 10^{-6} transductants per plaque forming unit (p.f.u.) for penicillin-V-sulfoxide and about 3 x 10^{-7} transductants per p.f.u. for tetracycline and erythromycin.

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The penicillin-V-sulfoxide resistance transduction frequency is similar in magnitude to the frequencies observed with P1 transduction in E. coli (Wall and Harriman, 1974) or P22 transduction in S. typhimurium (Schmieger, 1972). The frequency suggests the relatedness of the indicator strains' chromosomes.

Bacterial pathogens which are clinical isolates were selected on the basis of their MIC values, the highest for each antibiotic; tetracycline, penicillin-V-sulfoxide and erythromycin of 145, 270 and 200µg/ml respectively exceeded the clinical therapeutic dosages of 36, 36 and 144.9µg/ml used in the inhibition of Pseudomonas aeruginosa, S. group D and Proteus species respectively (Trounce *et. al.,* 1973).

The MIC values of the clinical isolates were also compared to the values of the increased tolerance levels of antibiotic by the bacteria after induction of resistance and the latter were found to be very high (Figs. 8) evident of the several cases of complications, arising in the treatment of such bacteria-related diseases. The abuse of these antibacterial agents by people who administer such drugs, without the requisite prescription and their use in animal feed as growth promoters have contributed significantly to this problem.

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4.1. 2 CONCLUSION

The ten phages that were isolated and partially characterized have similar characteristics with T-even and P phages. Restriction fragment lengths of their DNA have molecular weights ranging from 0.125 to 33.113kbp and the fragment of molecular weight 23.000kbp was common to nine of the phages, suggesting a relationship between their nucleic acid. The apparent molecular weight range of the polypeptide bands of the ten phages was between 11.7 and 199.5kd and also the polypeptide bands of apparent molecular weight range of between 12.9 and 14.8kd was common to all the ten phages, indicating a critical polypeptide in the expression of the phage activity and or morphology. Electron micrographs confirmed that nine of the phages had similar morphologies. The rate of inactivation, K, for EcRCL24 of 988 and the corresponding K values for the other EcRCL phages shows an appreciable cross reactivity and they are therefore closely related to EcRCL24. From the transduction experiments, the phages are presumed to be capable of generalized transduction and the frequencies of 3×10^{-6} for penicillin-V-sulfoxide and 3×10^{-7} for tetracycline and erythromycin are indicative of the relatedness of the indicator strains chromosomes.

Also the additional information which fell within the scope of the project, involving comparison of the MIC values of the clinical isolates to their increased tolerance levels of antibiotic after induction of resistance, shows that the clinical isolates can tolerate very high dosages of these antibacterial agents.

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A critical analysis of the electron micrographs of the phage samples shows that their morphologies do not determine the host range and this observation is significant in our understanding of phages.

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