Research Commons

http://researchcommons.waikato.ac.nz/

Research Commons at the University of Waikato

Copyright Statement:

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

The thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of the thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from the thesis.

THE CHARACTERISATION OF THE CATIONIC ANTIBACTERIAL COMPOUNDS FROM SOME MAMMALIAN TISSUES AND SECRETIONS

A thesis

submitted in partial fulfilment

of the requirements for the Degree

of

Doctor of Philosophy in Biochemistry

at the

University of Waikato

by

PHILLIP JOHN SHACKELL

University of Waikato

1984

ABSTRACT

Non-specific antimicrobial substances occur in mammalian tissues and secretions which are involved in the protection of the host against invading microorganisms. Among them are well researched proteins like lactoferrin and lysozyme, and systems like the myeloperoxidase- and lactoperoxidase-mediated system, and the two complement systems. Also, a number of mammalian cationic proteins have been found to have antibacterial properties. Conflicting data exist on their identity, as most of them have not been purified to homogeniety.

This investigation was designed to find out if any of the cationic heterogeneous antibacterial proteins extracted from mammalian tissues and fluids by various workers owe their activity to the polyamine spermine which was found in bovine rumen by Briggs (1982).

Previously Eschenbruch (1980) had isolated a low molecular weight antibacterial compound from bovine thymus, spleen and seminal plasma as well as sheep thymus and found them to behave similarly on cationic electrophoresis to the low molecular weight compound from bovine rumen. This was later identified as spermine (Briggs, 1982). Spermine was found to be associated with a substance which was responsible for absorbance at 210 nm.

Initially beta-lysin from bovine serum was investigated for the presence of spermine. Beta-lysin was found not to contain spermine so further purification of the antibacterial substance was undertaken. A homogeneous cationic protein was isolated which had an estimated molecular weight of 6 100.

Also, an investigation into the antibacterial seminalplasmin preparation found that no spermine was associated with this protein. However, further purification of seminalplasmin resulted in the finding that it had a lower molecular weight than that published (7 500 rather than 10 600). A lysozyme-like substance similar to that isolated by Eschenbruch (1980) was found closely associated with the seminalplasmin during the purification procedure.

The antimicrobial milk cell cationic proteins were also investigated. Although spermine was not found associated with the milk cell proteins, further isolation and purification of the active components was carried out. The milk cell proteins exist in a highly aggregated state and normal chromatographic techniques and eluents proved unsuccessful in isolating the active compounds. However EDTA was found to be a useful disaggregating agent. Two antibacterial proteins were isolated and were found to have molecular weights of 11 000 and 18 000-20 000. The higher molecular weight protein had an isoelectric point greater than pH 9.5 and was able to lyse pregrown Micrococcus lysodeikticus cells. The lower molecular weight protein gave an anomalous results on isoelectric focusing and displayed no lytic activity. There did not appear to be any interaction between the two proteins as far as enhancing or inhibiting their overall antibacterial activities.

An investigation into a bactericidal sheep thymus preparation resulted in the isolation of spermine and spermidine. This had been previously isolated by Eschenbruch (1980) and found to be electrophoretically similar to the bovine rumen antibacterial peptide. Further investigation of the antibacterial peptide found associated with lysozyme (which was also found to be electrophoretically similar to the

bovine rumen antibacterial peptide) did not contain spermine. Isolation of the active factors using a variety of dissociating agents was not successful as the active compounds were highly aggregated. There appeared to be several antibacterial compounds present with molecular weights estimated to be below 15 000.

The significance of the isolated cationic proteins are considered with regard to the non-specific defence against infections. Also the role of spermine and spermidine as a non-specific antibacterial agents is discussed along with the possibility that the cationic substances are artifacts produced through proteolysis of larger peptides or proteins as a result of the methods of extraction.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr P.C. Molan, for his guidance during the practical work and constructive criticism during the writing of this thesis.

I would like to thank Dr D. Cowan for his assistance as well as Mary Smith, Lyn Briggs and my fellow students.

I also wish to thank my family for their encouragment throughout.

Finally, I am indebted to the Meat Industry Research Institute of New Zealand for a Postgraduate Research Fellowship.

TABLE OF CONTENTS

Abstract ii

Acknowledgements v

Table of Contents vi

CHAPTER 1

INTRODUCTION 1

CHAPTER 2

MATERIALS AND METHODS 23

2.1 Tissues and Secretions 23

2.2 Dialysis 24

2.3 Rotary Evaporation 24

2.4 Buffers 24

2.5 Antibacterial Assays 24

2.6 Gel Filtration Chromatography 26

2.7 Ion Exchange Chromatography 27

2.8 Adsorption Chromatography 28

2.9 Thin-layer Chromatography 28

2.10 Acid Hydrolysis 28

2.11 Cationic Electrophoresis 29

2.12 SDS Electrophoresis 31

2.13 Gel Stains 31

2.14 Isoelectric Focusing 33

2.15 Protein Standards 33

CHAPTER 3

BETA-LYSIN 35

3.1 Introduction 35

3.2 Properties of Beta-lysin 36

3.3 Isolation of Beta-lysin from Bovine Blood 37

3.4 Purification of Bovine Beta-lysin 43

CHAPTER 4

SEMINALPLASMIN 50

4.1 Introduction 50

4.2 Properties of Seminalplasmin 50

4.3 Isolation of Seminalplasmin 51

4.4 Testing for the Presence of Spermine 59

4.5 Further Purification of Seminalplasmin 61

4.6 Estimation of Molecular Weights of the Antibacterial Proteins in Seminal plasma 68

4.7 Determination of Lysozyme Activity 71

4.8 Determination of Isoelectric Points 72

4.9 Conclusions 72

CHAPTER 5

THE CHARACTERISATION OF THE ANTIBACTERIAL COMPOUNDS FROM THE CELLS IN MILK 75

5.1 Introduction 75

5.2 Some Properties of the Isolated Antibacterial Cationic Proteins 76

5.3 Extraction and Identification of the Cationic Proteins from Milk Cells 77

5.4 Investigation of the Presence of Spermine in the Milk Cell Extract 78

5.5 Isolation of the Active Components from the Milk Cell Extract 85

5.6 Gel Filtration Chromatography in the Presence of SDS 87

5.7 The Use of Preparative Electrophoresis and Isoelectric Focusing in the Separation of the Antibacterial Components 94

5.8 Gel Filtration Chromatography Using Various Dissociating Agents as Eluents 111

5.9 Purification of the Antibacterial Compounds from the Milk Cell Extract 115

5.10 Molecular Weight Determination of the Two Antibacterial Proteins 124

5.11 Isoelectric Point Determination of the HMW and LMW Proteins 125

5.12 Determination of Lysozyme Activity 129

5.13 Interaction Between the HMW and LMW Proteins in their Antibacterial Activities 130

5.14 Conclusions 130

CHAPTER 6

INVESTIGATION OF THE SHEEP THYMUS PEPTIDE 133

6.1 Introduction 133

6.2 Properties of the Antibacterial Preparation Described by Hirsch and Dubos (1954) 134

6.3 Characterisation of the Antibacterial Activity from Bovine Thymus 135

6.4 Isolation and Identification of the Sheep Thymus Peptide 136

6.4.1	Isolation of Spermine	138
6.4.2	Investigation of the Component of Higher Molecular Weight	141
6.4.2.1	Investigation of the Peptide Fraction from the Component of Higher Molecular Weight	143
6.4.2.2	Investigation of the Spermine Content of the Peptide Fraction from the Component of Higher Molecular Weight	144
6.4.2.3	Attempted Separation of the Antibacterial Compounds from the Peptide Fraction	146
6.4.2.3.1	Separation of Fractions 8-11	149
6.4.2.3.2	Separation of Fractions 12-18	151
6.4.2.3.3	Separation of Fractions 19-22	156
6.5	Further Attempts at Isolating the Antibacterial Compounds from the Sheep Thymus Preparation	162
6.6	Isolation of the Sheep Thymus Antibacterial Proteins Using EDTA	177
6.7	Further Purification Methods Attempted	192
6.8	Conclusions	209

CHAPTER 7

DISCUSSION 211

REFERENCES 233

CHAPTER ONE

INTRODUCTION

Since the turn of the century researchers have frequently demonstrated the existence of naturally occurring non-specific antimicrobial substances in mammalian tissues and secretions. There is a great deal of confusion regarding the identities of these substances and their role supplementing the immune response in natural resistance to invading microorganisms. A great deal of this confusion is due to the lack of characterisation of these various antimicrobial agents as they are very difficult to obtain in a homogeneous state. The non-specific natural defence mechanisms involved in the protection of the host against invading microorganisms have been reviewed by Eschenbruch (1980) and Shackell (1980).

One of these mechanisms is the phagocytic defence system. After ingestion of a microorganism a number of antibacterial factors within the phagocytic vacuole can lead to the killing and lysis of the parasite. Among these factors are low pH, lactic acid, hydrogen peroxide, the myeloperoxidase-mediated system, cationic proteins, lysozyme and lactoferrin (Hirsch, 1960a, 1972). Some of these substances are also present in body fluids such as saliva, tears, milk, blood and seminal plasma. Blood also has the complement system as an additional antibacterial constituent.

Many of the non-specific substances have been found to be cationic peptides and proteins. One of the best characterised cationic proteins is lysozyme. It is found in tears, nasal mucus, saliva, blood serum and plasma, in many other tissues and secretions of human and animal origin,

in microorganisms, plants and egg white. Egg white lysozyme has an isoelectric point of 10.5-11.0 and a molecular weight of 14 700 (Salton, 1957).

Lysozyme is very stable under acidic conditions and withstands heating at 100°C at pH 4.5 for two minutes without losing activity. It is, however, labile at alkaline pH. Human lysozyme with three cystine residues per molecule is a little more heat-sensitive, and in goose lysozyme, which only contains two cystine residues, the heat stability is almost lost (Jollès, 1969).

Lysozyme exhibits both enzymic and non-enzymic action. As an enzyme, lysozyme specifically attacks the beta-1-4 glycosidic linkage that joins N-acetyl muramic acid and N-acetyl glucosamine in the murein backbone of peptidoglycan (Strominger and Ghuysen, 1967). Therefore, to be active, lysozyme must reach the critical glycosidic bonds and cleave a sufficient number to result in fracture of the murein backbone of the peptidoglycan.

The non-enzymic action of lysozyme involves flocculation of bacterial suspensions owing to the basic properties of the protein. Other basic substances such as protamine, histones and basic polypeptides do this also. Negatively charged groups on the bacterial surface are likely to attract the basic compounds thus causing neutralisation of the charges and agglutination of the cells (Salton, 1957).

The role of lysozyme in resistance to bacterial infections has never been clearly established (Skarnes and Watson, 1957). While most bacteria of clinical significance are resistant to lysozyme itself, the major function of this enzyme may be to act in concert with other

factors (Root and Cohen, 1981). Lysozyme had been shown, for example, to act cooperatively in killing organisms with the serum antibody--complement system (Muschel and Jackson, 1963), with a combination of hydrogen peroxide and ascorbate (Miller, 1968), and with both high and low pH (Saint-Blancard et al., 1970). Elsbach (1980) suggested that the most important role of lysozyme in neutrophil function may be to digest phagocytosed bacteria once they are killed rather than to act as a primary bactericidal agent. However Spitznagel (1979 and 1980) supports the view that lysozyme plays an important role in killing as well as degradation of susceptible bacteria.

As well as lysozyme, there are other bactericidal agents present in mammalian serum. The bactericidal action of serum for Gram-negative bacteria is mediated by the antibody-complement system. A serum bactericidal agent effective against Gram-positive bacteria has been termed beta-lysin (Pettersson, 1924). Donaldson and Marcus (1958) demonstrated that blood coagulation mechanisms exert a mediating effect on the serum bactericidal activity, and Hirsch (1960b) established that beta-lysin originates from blood platelets.

Blood coagulation was shown to be essential for the liberation of beta-lysin from the platelets. Recently Tew and coworkers (1974) demonstrated that thrombin is involved in the release of beta-lysin. Inflammatory exudates are also rich in free beta-lysin, indicating its involvement in infections (Jenson et al., 1967).

Beta-lysin is a cationic protein with a molecular weight of 6,000 (Johnson and Donaldson, 1968). It is heat-stable, remaining active after incubation at 97°C for 30 minutes. According to Johnson and Donaldson there are other substances like beta-lysin in serum. One of

them, which they called staphylocidal beta-lysin, is very similar to the original beta-lysin: it is heat-stable, of similar molecular weight and can be isolated using almost the same techniques, yet it differs in several other aspects and is not released from platelets.

Recent investigations (Carroll and Martinez, 1979b) reported that purified beta-lysin isolated from normal rabbit serum by the classical procedure is a heterogeneous mixture of compounds. They also demonstrated that the kinetics of action, cellular source and relative concentrations of serum bactericides (beta-lysins) vary between animal species.

The cell membrane is considered to be the primary site of action of beta-lysin as it combines with purified cell membranes (Gooch and Donaldson, 1974), and also prevents the development of the space between the wall and the plasma membrane which normally occurs in cells suspended in hypertonic sucrose solutions (Matherson and Donaldson, 1968). Beta-lysin was also found to rupture protoplasts (Matherson and Donaldson, 1970).

Other microbicidal mechanisms which have recently been investigated are those from alveolar macrophages. Their function in host defence is well known and a recent review by Hocking and Golde (1979) reports on the microbicidal proficiency of alveolar macrophages.

Lehrer _et al_. (1980) demonstrated that the mechanism(s) whereby rabbit macrophages killed _Candida albicans_ differed substantially from the myeloperoxidase-hydrogen peroxide-mediated antifungal systems predominantly operative in human monocytes and neutrophiles. Rabbit granulocytes have already been shown to contain microbicidal lysosomal cationic proteins (Zeya and Spitznagel, 1968, 1969, and 1971).

Macrophages and granulocytes are thought to arise from a common stem cell, and contain certain constituents in common (e.g. lysozyme).

Patterson-Delafield et al. (1980) reported the isolation of two highly cationic microbicidal proteins from rabbit alveolar macrophages. They were shown to be distinct from histones and not to arise from granulocyte contamination. Both microbicidal cationic proteins 1 and 2 (MCP-1 and MCP-2) were highly active against diverse Gram-positive and Gram-negative organisms under conditions of near neutral pH and relatively low ionic strengths (Lehrer et al., 1983). Molecular characterisation of the peptides revealed them to be small, rich in arginine and cystine, and devoid of carbohydrate or reduced sulphydryl groups (Patterson-Delafield et al., 1981). Each peptide is composed of 33 amino acid residues. MCP-1 differs from MCP-2 only by containing arginine instead of leucine at the 13th position from the amino acid terminus (Selsted et al., 1983). Of the various bacterial species studied, only B. bronchiseptica appeared to be resistant to MCP-1 and MCP-2. As bronchopulmonary colonisation of rabbits by B. bronchiseptica is extremely common they suggested that the resistance of this microorganism to MCP-1 and MCP-2 may be an important factor allowing its persistence in the rabbit respiratory tract.

Other studies show that S. aureus is not killed by alveolar macrophages unless the bacterial cells are first exposed to alveolar lining material (LaForce et al., 1973). They demonstrated that it is the surfactant-containing fraction of alveolar lining material that enhances intracellular killing of S. aureus. Coonrod and Yoneda (1983) also demonstrated that the alveolar lining material from rats caused rapid killing and lysis of pneumococci. Killing by the surfactant fraction was associated with increased bacterial cell membrane

permeability. It was suggested that the surfactant fraction may be a lysophospholipid, palmitoyl lysophosphatidylcholine. It has many similar properties such as antibacterial activity against several Gram-positive bacteria and alters cell membrane permeability.

Many antibacterial cationic proteins have also been isolated from polymorphonuclear leukocyte granules. They have dissimilar antibacterial spectra and vary from one species of animal to another, differing in molecular weight, amino acid composition, and enzymic properties. The human cationic proteins range in molecular weight from 10 000 to 25 000 (Odeberg and Olsson, 1975). In contrast to the human proteins, the three most cationic proteins from rabbit polymorphonuclear leukocytes ranged from 4 000 t0 8 000 (Zeya and Spitznagel, 1968).

Extracts of cationic proteins from rabbit polymorphonuclear leukocytes that kill E. coli by increasing the permeability of the microbial envelope have been reported by another group of research workers (Berkerdite et al., 1974; Weiss et al., 1975,1976). Purification and characterisation of the bactericidal protein had been described and compared with a cationic protein isolated under the same conditions from granules of human polymorphonuclear leukocytes (Weiss et al., 1978; Elsbach et al., 1979). These proteins have been named "bactericidal/permeability-increasing protein" (BPI). The molecular weight of human BPI is 59 000 and of rabbit BPI is 50 000. BPI is larger than any previously described antibacterial protein isolated from neutrophil granules. Both proteins are strongly basic (isoelectric points greater than 9.6) and relatively heat-resistant. The amino acid compositions of the two proteins are very similar.

The antibacterial actions of BPI are highly specific for certain Gram-negative bacterial species. This specificity seems to be closely related to the bacterial envelope structure that determines whether or not, and with what degree of affinity, BPI binds to bacterial surface structures (Elsbach and Weiss, 1981, 1983). Binding is an absolute requirement for the action of BPI (Weiss et al., 1980), but is not sufficient to generate the antibacterial effects of BPI (Weiss et al., 1983). Apparently, saturation binding is required to trigger "postbinding" steps that are actually responsible for the membrane-active and bactericidal effects of BPI. The hydrophobic interactions with the outer membrane irreversibly trigger the bactericidal action of BPI. The BPI can be removed from the bacterial surface by 80 mmol/l $MgCl_2$ or by trypsin. This results in repair of the envelope alteration, but viability is irreversibly lost even after only 15 seconds exposure to BPI (Elsbach et al., 1983). This suggested that BPI is the principle bactericidal agent in the intact neutrophil against E.coli and S. typhimurium.

A new protein with bactericidal activity was discovered by Spitznagel and colleagues from leukemic polymorphonuclear leukocytes. The extract, fractionated on a column of Sephadex G 100, eluted a highly active fraction in a region of low protein concentration (Modrzakowski et al., 1979). Further studies of granule extracts from normal human polymorphonuclear leukocytes revealed differences in the chromatographic fractions from Sephadex G 100 (Modrzakowski et al., 1981). The physicochemical properties of the granule extracts have not yet been described.

Hodinka and Modrzakowski (1983) separated extracts of rat polymorphonuclear leukocyte granules by column chromatography on Sephadex G 100 into three major peaks. The peak of lowest molecular weight (peak c) contained lysozyme and two components more cationic than lysozyme on cationic electrophoresis. The peak c fraction was heat-stable, maintaining bactericidal activity after boiling for 30 minutes. This is consistent with the findings of Walton (1978) and Odeberg and Olsson (1975) who reported the existence of heat-stable cationic proteins from rabbit and human polymorphonuclear leukocytes. Treatment with trypsin substantially abolished the antibacterial activity, suggesting the component(s) are of a protein nature.

The importance of cationic proteins may vary between animal species but nonetheless they have an important function. The weight of evidence suggests that cationic proteins of different kinds play a role in the antibacterial activities of polymorphonuclear leukocytes (Spitznagel, 1980).

A number of antibacterial cationic proteins have also been isolated from bovine teat canal keratin by acid extraction of the keratin (Hibbitt and Cole, 1968; Hibbitt et al., 1969; Hibbitt, 1970). After further purification, electrophoresis resolved the isolated proteins into six principal bands at pH 3.0. The cationic proteins inhibited the growth of mastitis-causing strains of staphylococcus and streptococcus, causing marked changes in the cell wall and plasma membrane. Similar changes were also seen in staphylococci recovered from the teat canal of a healthy cow, and in the presence of calf thymus histones (Macmillan and Hibbitt, 1969).

Cationic proteins with antimicrobial activity were also isolated from the cells in bulk milk samples (Hibbitt et al., 1971). Polyacrylamide gel eletrophoresis studies showed at least nine components with isoelectric points between 7 and 9 and one component with an isoelectric point above 9. The antimicrobial activity was not destroyed after heating at temperatures up to 70°C for 30 minutes, whereas at higher temperatures the activity diminished and was almost completely lost at 100°C. The cationic proteins isolated from milk cells had a greater antimicrobial activity than similarly charged proteins isolated from teat canal keratin.

An antimicrobial extract had also been obtained from normal bovine whey. This heat-stable, dialysable fraction was capable of killing several species of mycoplasmas (Brownlie et al., 1974; Howard et al., 1975). They distinguished it from other bovine antibacterial agents such as lactenin (Wilson and Rosenblum, 1952), the cationic protein fraction from bovine teat canal keratin (Hibbitt et al., 1969), the basic fraction present in cervical mucus (Brownlie and Hibbitt, 1972), and lactoferrin on the basis of dialysability and heat stability. However, the lack of further characterisation makes comparison with other antimicrobial substances difficult.

There are various antibacterial substances which have been identified in other mammalian tissues and secretions. In seminal plasma alone there are the amines spermine and spermidine, the proteins lysozyme, complement, seminalplasmin, and the prostate antibacterial fraction.

The presence of lysozyme in bovine seminal plasma has been demonstrated (Eschenbruch, 1980). However the presence of lysozyme in the secretions of the human male reproductive tract has not been convincingly demonstrated (Shivaji, 1984).

In experiments with human semen it was reported that antibacterial activity present in the system was due to spermine and spermidine (Rozansky et al., 1949; Gurevitch et al., 1951; Razin and Rozansky, 1957). The antimicrobial role of spermine in vivo was viewed by Hirsch (1960a) as speculative since spermine in tissues may be firmly bound or its action on microorganisms may be influenced by the presence or absence of other material in the body. Tabor et al. (1961) also showed that an alkaline medium is needed for optimum activity. Fair and Wehner (1971) studied the effects of spermine against a variety of microorganisms in an effort to determine the role of spermine in the human male. They demonstrated that spermine would have little if any effect against the majority of organisms normally responsible for urinary tract infections, at the normal acidic pH of prostatic secretions.

A fraction possessing antibacterial activity against Gram-negative and Gram-positive bacteria was isolated from canine prostatic fluid by Stamey et al. (1968). On gel filtration of the prostatic fluid on Sephadex G 10, the proteins of high molecular weight were eluted in a single peak that had no antibacterial activity. Another peak of proteins, named the prostatic antibacterial fraction (PAF), was eluted after the large peak. PAF had no lysozymal activity and was unrelated to spermine. Fair et al. (1973, 1976) further studied the antibacterial factor of human and canine prostatic fluid. They determined its molecular weight to be 1 500 or below, and also showed it

to be a potent bactericidal cationic substance which is heat-stable and water-soluble. They also found that 90% of the organisms responsible for urinary tract infection were sensitive to this antibacterial agent. In further work, the prostatic antibacterial factor (PAF) responsible for the activity of normal prostatic fluid was identified as free zinc (Fair *et al*., 1976); Fair and Parrish, 1981). They suggested that the bactericidal activity of the prostatic secretions is related to the amount of zinc present in the fluid and may play a role in the natural resistance of the male urinary tract to infections.

Another antibacterial agent isolated from bovine seminal plasma was seminalplasmin (Reddy and Bhargava, 1979). It was heat-stable, had as isoelectric point of 9.8 and a molecular weight of between 8 000 and 19 800 depending on the method used. Seminalplasmin was found to have potent antibacterial activity against a variety of Gram-positive and Gram-negative bacteria. They showed that the microbicidal action of seminalplasmin was due to the inhibition of RNA synthesis. Protein synthesis in *E. coli* started to decline linearly 10 minutes after incubation with seminalplasmin. They showed that r-RNA and t-RNA were inhibited 85% and 10% respectively. Scheit *et al*. (1979) extended the investigations and found that seminalplasmin was also a potent inhibitor of RNA polymerase of *E. coli*. It bound strongly to the enzyme and caused inhibition of transcription of up to 90% at a concentration of 5 nmol/ml. The heating of seminalplasmin to 80°C for 10 minutes had no effect on the bactericidal activity against *E. coli*, whereas 90% of the transcription-inhibiting activity was destroyed.

Reddy et al. (1983) also showed seminalplasmin to be a potent inhibitor of several reverse transcriptases, purified or in viral lysates. Seminalplasmin appears to be the first purified protein shown to be an inhibitor of reverse transcriptases. They suggested that seminalplasmin may play a role in the protection against retrovirus of the male or the female reproductive tract, or the fertilised ovum.

Shannon et al. (1974, 1975) have also studied the antibacterial activity of seminal plasma obtained from vasectomised bulls. The activity was heat-stable and dialysable at pH 3 but not at pH 7. Consequently the material was described as being heterogeneous, existing in various forms ranging from a monomer to polymers of large molecular weight. Shannon et al. (1975) suggested that the association of the peptide with larger proteins may be advantageous, and would perhaps be less susceptible to inhibition by anionic substances. Preliminary characterisation by Schollum et al. (1977) of the antibacterial factor in bovine seminal plasma confirmed the heat stability, and showed it to be destroyed by some proteolytic enzymes.

Shannon et al. (1975) also found antibacterial activity in cell-free extracts of pancreas, spleen, liver and lungs, which had been dialysed for 15 days against water at pH 7.0 before fractionation. Bactericidal activity was also obtained from bovine kidney, saliva, intestinal mucosa, leukocytes and teat canal epithelium dialysed at pH 3.0. All dialysates were found to have a major component with the same electrophoretic mobility as the peptide from seminal plasma. The substance was shown by gel filtration chromatography at pH 1.7 or 12.0 to have a molecular weight of 3 500. On examination of several bovine tissues, Molan (personal communication) found that rumen yielded large quantities of the low molecular weight antibacterial factor in the most

disaggregated form. He developed a small-scale method of extraction which produced small quantities of this substance free from larger proteins.

Shackell (1980) continued this study and developed a method of extraction on a larger scale for isolation of the factor from bovine rumen. The molecular weight of the antibacterial peptide estimated on thin layer gel filtration chromatography was found to be from 2 000 to 3 200 depending on the eluent used. The antibacterial peptide was found to be heat-stable (1 hour at 100°C), sensitive to trypsin and chymotrypsin and inactive in the presence of 0.7 mol/l NaCl. Attempts to estimate the isoelectric point using isoelectric focusing were unsuccessful. Further efforts using polyacrylamide gels at various high pH values, to obtain a change in direction of migration after the isoelectric point was reached were also unsuccessful, it being cationic at pH 13.

The rumen antibacterial peptide and samples obtained by Eschenbruch (1980) from bovine seminal plasma, spleen and thymus were submitted to cationic electrophoresis. They displayed very unusual behaviour in that they were detected on the surface of the gel, and were therefore initially lost during destaining procedures.

Briggs (1982) studied the antibacterial peptide further and found that the substance responsible for the antibacterial activity in the rumen preparation was the polyamine, spermine. On gel filtration chromatography, spermine was found to be associated with a substance which was responsible for absorbance at 210 nm. This material could be dissociated from spermine by the formation of spermine phosphate or by ion exchange chromatography.

Polyamines have been reported to be ubiquitous in biological materials but are found in highest concentrations in tissues that actively synthesize protein and have a high RNA content (Tabor and Tabor, 1964). Considerable variations in polyamine levels have been found in different animal tissues and fluids (Bachrach, 1973) and different species, but particularly high concentrations have been reported in the pancreas, prostate gland and human semen. Polyamine levels within these tissues and fluids may vary with physiological variations.

Early work on polyamines left many biochemists with the impressions that these cations were the end product of a degradative pathway and that the instances of polyamine occurrence in mammalian systems were keyed to bacterial decay or to excretion into seminal fluid. Hämämläinen in 1947 performed an analytical study of the spermine content of normal and diseased human tissues. In cases of pneumonia, tuberculosis, and carcinoma, the diseased tissue consistently contained approximately twice as much spermine as a normal portion of the same organ. This pointed to the possible biological significance of these compounds (Tabor and Tabor, 1964).

Among the first physiological effects ascribed to polyamines was the ability to stabilise bacteria and other cells. Mager (1955) demonstrated that *Pasteurella tularensis* which loses viability when washed with distilled water, was protected by using saline or water containing spermine. The stabilisation of protoplasts and membranes of *Streptococcus faecalis* was also demonstrated by Harold (1964) using spermine and spermidine at a concentration of 0.001 mol/l. It was thought that the stabilisation of protoplasts resulted from ionic binding of the cation to acidic sites on the external surface of the

plasma membrane. Similarly, Grossowicz and Ariel (1963) reported that spermine and spermidine will prevent lysozyme from lysing *Micrococcus lysodeikticus* by protecting the membrane but not by inhibiting the action of lysozyme. They suggested that spermine penetrated through the cell wall and was tightly bound to the cytoplasmic membrane. The high concentration of phospholipids in the membranes of bacteria, protoplasts and mitochondria suggest the possibility that the formation of polyamine-phospholipid complexes may account for the effect of polyamines on membrane stability (Tabor *et al*., 1961). Stabilisation by spermine has also been described for subcellular particles such as nuclei, nucleoli, mitochondria and ribosomes (Bartos and Bartos, 1978).

Polyamines have also been shown to stabilise nucleic acids against denaturation and shearing (Cohen, 1971; Bachrach, 1973). Tsuboi (1964) and Liquori *et al*. (1967) have shown that spermine stabilises DNA in solution and have suggested that it does this by forming links across the narrow groove. There is now increasing evidence that the ability of polyamines to influence the conformation of the nucleic acids plays a significant role in their effects on macromolecular synthesis (Cohen, 1971).

Stevens (1967) synthesised four homologues of spermine and found that they resembled spermine in their ability to stabilise *E. coli* protoplasts. This led to the conclusion that the stabilisation of the protoplasts is a non-specific effect independent of the chain length, unlike the stabilisation of DNA which was more specific.

Although the physiological roles of spermine and spermidine are still unknown, their activity as growth factors for certain microorganisms has been clearly demonstrated. Bachrach (1973) suggested

that polyamines facilitate growth by stabilising membranes thereby preventing lysis. However, Inouye and Pardee (1970), suggested that polyamines are required for bacterial cell division.

Polyamines have also been described as growth factors for mammalian cells. Polyamine function has been studied using polyamine-deficient cells (Morris, 1978). The results indicated that polyamines are required for cell proliferation. Atkins et al. (1975) first demonstrated that the addition of polyamines results in a qualitative difference in the polypeptides synthesised in a cell-free system. When the system was supplemented with polyamines, the polypeptides synthesised *in vitro* were larger and resembled the products *in vivo* more closely. Subsequently direct evidence was presented that polyamines enhanced peptide elongation (Abrahams et al., 1979). Igarashi and Hirose (1978) studied polypeptide synthesis in *E. coli* and rat liver cell-free systems. They found that the increase of polyphenylalanine synthesis by spermidine occurs at the level of aminoacyl-tRNA binding to ribosomes and not at the level of peptide bond formation and translocation. Polyamines may stimulate synthesis by overcoming defects in biosynthesis and/or assisting assembly of small ribosomal subunits (Holtta et al., 1979). On the other hand polyamines may somehow facilitate the movement of the ribosomes along the messengers and enable them to surmount obstacles occurring at specific sites. They probably do so by altering the secondary structure of the template and/or the conformation of t-RNA (Abraham and Pihl, 1980, 1981).

In contrast to their function as growth factors, spermine salts (15 mg/kg) have also been shown to cause acute renal tubular necrosis in mice, rats, guinea-pigs, rabbits and dogs (Rosenthal et al., 1952). Studies by Razin and Rozansky (1957) also indicated that antimicrobial

action present in semen could be attributed to the presence of spermine and spermidine. However the antibacterial activity present in prostate fluids and amniotic fluids was only partially due to these polyamines (Fair and Wehner, 1971; Miller et al., 1976). Detailed studies of the antimicrobial action of spermine and spermidine were carried out by Razin and Rozansky (1959). In general, spermine was found to be the most effective in inhibiting bacterial growth, spermidine being somewhat less active. Exponentially growing cultures of E. coli were studied after exposure to a lethal concentration of spermine (Mills and Dublin, 1966). The first changes noted were a slowing of protein synthesis, stimulation of RNA synthesis and increased potassium fluxes. This was followed by a decline in viable numbers. Polyamines have been also shown to inactivate certain viruses (Bachrach, 1973).

Razin and Rozansky (1959) also found that nucleic acids, lecithin, basic organic compounds and inorganic salts antagonise the antibacterial action of spermine. The antibacterial action of various drugs is inhibited by polyamines which suggests a common binding site. Such effects have been reported for streptomycin (Bragg and Polglase, 1963) and a number of other drugs.

Polyamines can also affect the cell surface of bacteria. This can happen either by direct attack on membrane permeability properties or by causing cell clumping by neutralising the polyanionic nature of the cell walls (Silver et al., 1970). It appears that the bactericidal effect occurs only in a nutritionally adequate medium. Glucose and a temperature of 37°C are required to inhibit growth (Razin and Rozansky, 1959). These results are consistent with the finding that energy is required for the binding of polyamines to microbial cells (Tabor and Tabor, 1966).

The biological functions of polyamines were also investigated according to their location within the cell (Bachrach, 1973). However conventional methods for preparing the different cell compartments probably resulted in leakage of polyamines from organelles or redistribution during the preparation. Recently a rapid method for the separation of cytoplasm and nuclei for polyamine analysis of the different compartments of BSC-1 cells has been developed (Mach et al., 1982). By separating the cytoplasm and the nuclei within 20 seconds they found strikingly different polyamine distributions in the cell compartments. In the nucleus spermine is present in a five-fold excess over putrescine and spermidine, whereas in the cytoplasm putrescine and spermine are present in almost equal amounts. These results supported the earlier hypothesis that spermine is associated with DNA.

The physiological significance of polyamines has been established, but no specific role had been firmly accepted for them in vivo. Cohen (1978) has suggested that as cations are essential for growth, then the ability to use polyamines to replace an essential divalent cation would provide a selective advantage in certain physiological conditions. Holtta et al. (1979) also saw polyamines as organic cations with concentrations which can be precisely regulated. Polyamines are involved in several metabolic processes and structural functions in the cell, with an absolute structural specificity required possibly only in a few specific cases. Jänne et al. (1978) agreed that most of the effects exerted by polyamines can be explained in terms of their cationic nature, and that they act intracellularly as nonspecific cations. However. not all effects can be ascribed to the chemical structure of these compounds. Alternatively Morris (1981) suggested these cations may be only involved in the bulk neutralisation of

intracellular negative charge, as for example that arising from nucleic acids and membrane phospholipids.

Most of the physiological functions which have been suggested for these compounds are dependent on the polyamine cationic charge. Each of the amine groups are positively charged at physiological pH. Polyamines can bind to carrier molecules by hydrogen bonding to form complexes, or by covalent bonding to form conjugates. This results in the net positive charge being reduced on the polyamine molecules which therefore modifies their function. Non-covalent bonds such as hydrogen bonds are relatively weak when compared with covalent bonds, and therefore complexes are usually able to be dissociated under ordinary conditions. However with spermine there are four amine groups available for hydrogen bonding. Therefore these bonds will help reinforce each other. Several proteins have been isolated which specifically bind spermine. Mezzetti et al. (1980) reported the existence of a selective spermine-binding protein in the cytosol fraction of chick duodenal mucosa. This protein was heat sensitive and had a molecular weight of about 32 000. More recently Mezzetti et al. (1982) found that human peripheral lymphocytes contain a cytosol factor able to bind spermine non-covalently and with a high affinity. This factor is heat sensitive and appears to be a protein which is an intracellular, specific binder for spermine.

Conjugates of polyamines are strongly bound by covalent bonding, hence extreme conditions are required for the total release of polyamine from these derivatives. A significant portion of the polyamine found in human urine is acetylated (Bachrach, 1976) although conjugation to peptide carriers has also been suspected (Seale et al., 1978). As well as acetyl conjugates many other polyamine derivatives have been found in nature. Edeine A and edeine B are antibiotics which were obtained from

Bacillus brevis Vm 4. They contained spermidine or guanylspermidine in covalent linkage to a pentapeptide (Hettinger et al., 1970)

Roch et al. (1979) have identified the existence of a polyamine conjugate of high molecular weight in which spermine is bound to immunoglobulin. The chemical structure of the spermine-immunoglobulin complex is not known. Other polyamine-peptide conjugates have recently been identified and their chemical structures defined. Chan et al (1979) found that essentially all of the putrescine and a significant amount of the spermine in human amniotic fluid exists in the form of polypetide conjugates. The putrescine-containing peptide has a molecular weight of 5 000 whereas the spermidine conjugate has a molecular weight between 10 000 and 30 000. Neither conjugate contains detectable amounts of the other polyamine.

Briggs (1982) extracted spermine from bovine rumen as a complex bound to a substance which was responsible for absorbance at 210 nm. The binding was noncovalent and due to ionic or hydrogen bonding. This spermine complex did not show any significant differences in the level of antibacterial activity to that of bound or free spermine. The spermine complex from bovine rumen had previously been isolated and thought to be a cationic peptide. The possibility that many of the heterogeneous antibacterial cationic proteins may owe their activity to the presence of spermine was considered.

Eschenbruch (1980) also studied a number of antibacterial extracts from several tissues and secretions. One of these extracts, the bovine and sheep thymus preparation (Dubos and Hirsch, 1954), a cationic antibacterial peptide (thymus peptide) and lysozyme was isolated. The thymus peptide could be separated from the thymus preparation in an

unbound form as well as from the lysozyme-containing fraction of high molecular weight. All of the antibacterial activity in these extracts was attributed to the presence of lysozyme and the thymus peptide. The thymus peptide was found to migrate similarly to the rumen peptide during cationic electrophoresis. However cationic electrophoresis was shown not to be a good method to use to compare small cationic peptides as they were all found to migrate similarly to spermine (Briggs, 1982). Therefore it was intended that the antibacterial peptide found by Eschenbruch (1980) in the sheep thymus preparation be investigated for the presence of spermine. If spermine was not found then the cationic proteins present would be examined and compared with other known cationic antibacterial proteins.

Other preparations that appeared similar were also investigated to see if their antibacterial activity was due to spermine. Shannon _et al_. (1975) found a cationic antibacterial peptide in bovine seminal plasma. This peptide forms aggregates of higher molecular weight without loss of activity. Fractionation of bovine seminal plasma by gel filtration chromatography resulted in active components with apparent molecular weights ranging from about 3 000 to 20 000. Eschenbruch (1980) also investigated bovine seminal plasma and isolated lysozyme as well as a cationic peptide. This cationic peptide had a migration rate on cationic electrophoresis similar to the bovine rumen peptide (subsequently found to be spermine). Reddy and Bhargava (1979) also isolated a cationic protein called seminalplasmin from bovine seminal plasma. The molecular weight of seminalplasmin was estimated to be between 8 000 and 19 800 depending on the method used. The discrepancy between these values may indicate a heterogeneous product containing the antibacterial peptide found by Eschenbruch (1980). Therefore

seminalplasmin preparation was investigated for the presence of spermine, with the intention of purifying to homogeneity if spermine were not present.

Other preparations that may have contained spermine were also investigated. The milk cell extract of Hibbitt and coworkers (1971) was examined as it contained a heterogeneous mixture of cationic proteins and it appeared possible that the preparation might contain spermine. Also, owing to the lack of characterisation of beta-lysin, as well as the report that purified beta-lysin isolated by the classical procedure is a heterogeneous mixture of compounds, it was investigated for the presence of spermine. If spermine was not responsible for the antibacterial activity in these preparations, then the compounds responsible were to be isolated. However Carroll and Martinez (1981a) subsequently described a procedure for the purification of beta-lysin from rabbit serum so the investigation of this was curtailed.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Tissues and Secretions

Bovine Seminal Plasma

Bovine seminal plasma was obtained from the NZ Dairy Board Artificial Breeding Centre, Newstead. Freshly collected samples from a number of vasectomized bulls were collected by means of an artifical vagina, pooled and stored frozen at -20°C.

Bovine Blood

Bovine blood was obtained from the Abattoir at the Ruakura Agricultural Research Centre, Hamilton, from freshly slaughtered cattle. Blood was collected in a bucket containing a 4% oxalate solution (12 g/l ammonium oxalate and 8 g/l potassium oxalate) added at a concentration of 50 ml/l of blood. The blood was immediately taken to the laboratory and processed.

Milk Cells

Bovine milk cells were collected from the Riverlea Road Milk Treatment Station, Hamilton. They were removed from the clarifier and immediately taken to the laboratory and processed.

Tissues

Frozen sheep thymus and bovine spleen were obtained from Auckland Farmers' Freezing Co-operative Ltd., Horotiu.

2.2 Dialysis

Dialysis was carried out using two types of dialysis tubing.

a) Thomas dialysis tubing with a molecular weight cut-off of 10 000 supplied by Arthur H Thomas Company.

b) Spectra/Por 3 dialysis tubing with a molecular weight cut-off of 3 500 supplied by Spectrum Medical Industries.

2.3 Rotary Evaporation

Samples were evaporated by rotary evaporation under vacuum using a Buchi Rotovapor, at a temperature of 35 to 40°C.

2.4 Buffers

Tris and phosphate buffers were prepared by the methods given by Dawson *et al*. (1969). Specific buffer concentrations and pH values are described in the individual experiments in the following chapters.

2.5 Antibacterial Assays

Routine testing was performed against an unidentified spore-forming bacterium similar to *Bacillus subtilis* (referred to hereon as *Bacillus* sp.), *Escherichia coli B* and in some cases against *Bacillus subtilis*. The bacteria were grown for 18 hours in 20 ml of Nutrient Broth (Difco Laboratories). This was then added to 500 ml of sterile nutrient broth containing 1% agarose (Sigma Chemical Company) at a temperature of about 45°C. Plates were poured immediately in a Laminar Flow Cabinet. The plates were stored for up to 3 weeks at 4°C.

Wells, 7 mm in diameter, were punched in the plates. The wells were numbered and filled with the appropriate test samples. The bacterial plates were then incubated at 37°C for about 18 hours. The extent of the zone of bacterial growth inhibition was measured in millimetres with a ruler. Data was expressed as the distance between the edge of the well and the edge of the clearing.

Measurement of lytic activity was based on the lyso-plate method of Osserman and Lawler (1966) where the activity of a sample is assayed by the lysis of pre-grown *Micrococcus lysodeikticus* cells suspended in nutrient agar plates.

An inoculum of *Micrococcus lysodeikticus* in 10 ml of nutrient broth was incubated at 37°C for 36 hours. It was then added to 100 ml of sterile Nutrient Agar (BBL) at about 45°C and immediately poured into petri dishes.

It was found that the agar mixture had to be as cool as possible before addition of the inoculum, as the *Micrococcus lysodeikticus* cells were very sensitive to heat.

The plates were incubated at 37°C for 18 hours after which time a bacterial growth had covered the plates. The plates were stored at 4°C for no longer than 2 weeks. Lytic activity was tested by the well diffusion technique as described earlier. After incubation for 12-18 hours, the size of the clear zones, formed by the lytic action of lysozyme, gave an indication of the concentration of lysozyme in the sample.

When testing samples with low lysozyme concentrations, 1% agarose plates containing 0.02% dried Micrococcus lysodeikticus cells (Sigma Chemical Company) were used as described by Gosnell et al. (1975).

These methods were used primarily for the location of lysozyme activity in fractions eluted from gel filtration and ion exchange columns. No attempt was made to quantify the assay.

2.6 Gel Filtration Chromatography

Gel filtration chromatography was carried out using "Sephadex" (Pharmacia) cross-linked dextrans of various pore sizes. Columns of Sephadex were prepared and used according to the instructions given in the booklet "Sephadex Gel Filtration in Theory and Practice", published by Pharmacia. If not stated otherwise, Pharmacia K 26 chromatographic tubes were used, with an internal diameter of 2.6 cm and lengths varying from 20 cm to 100 cm. Void volumes (Vo) and bed volumes (Vt) are marked in figures showing the elution profiles. The void volume was determined using a marker of high molecular weight such as blue dextran. Columns were eluted by upward flow using a peristaltic pump, the flow rates being 80 ml/h for Sephadex G 15 and G 25, 60 ml/h for Sephadex G 50 and 50 ml/h for Sephadex G 75. The composition of elution buffers is described with the individual experiments in the following chapters.

When using sodium dodecylsulphate (SDS) in gel filtration chromatography, it was found necessary to use Sepharose CL-6B, owing to the increased molecular size of the SDS-denatured proteins. The flow rate used was 50 ml/h.

The absorbance of the eluent was continuously monitored at a wavelength of 220 nm using a Cecil 272 Spectrophotometer and recorded on an Omniscribe chart recorder. Fractions were collected with an automatic fraction collector (LKB "Ultrorac" Model 7000). The fractions were marked on the chart by means of an event marker connected to the fraction collector.

High pressure liquid gel permeation chromatography was also used. Separation was achieved on a 21.5×600 mm preparative TSK G3000SWG column (Toya Soda Manufacturing Company) with a molecular weight separating range from 10 000 to 500 000. The flow rate was maintained at 3 ml/min by a Waters M-45 Solvent Delivery System. The absorbance of the eluent was continuously monitored at a wavelength of 220 nm using a Waters Lambda-Max Model 481 LC Spectrophotometer. Fractions were collected with an automatic fraction collector as detailed above.

2.7 Ion Exchange Chromatography

Ion exchange chromatography was performed in Pharmacia chromatographic tubes, type K 16/20, which have an inner diameter of 1.6 cm and a length of 20 cm. The ion exchange gel was packed into the columns giving approximate bed volumes of 30 ml. SP Sephadex C 25, with sulphopropyl functional groups, and CM Sephadex C 25 and C 50, with carboxymethyl functional groups, were used for ion exchange chromatography of basic proteins. For anion exchange chromatography, QAE Sephadex A 25, containing diethyl-(2-hydroxypropyl)aminoethyl groups, was used. Buffers and conditions used for eluting the columns are described with the experiments in the following chapters.

2.8 Adsorption Chromatography

Adsorption chromatography was performed in Pharmacia chromatographic tubes, type K 16/20. Bio-Gel HTP (Bio-Rad) was prepared and used according to the instructions in the current Bio-Rad catalogue. The eluent was monitored and collected as mentioned in section 2.6. The buffers and conditions used for eluting the column are described with the experiments in the following chapters.

2.9 Thin-layer Chromatography

Silica gel (Kieselgel C, Merck) was spread on chromic acid-cleaned plates (5×20 cm and 10×20 cm) using a Shandon Uniplan spreader. The gel thickness was 0.3 mm. The plates were activated by heating at 110°C for 30 minutes and cooled in a desiccator before use.
The following solvent system was used:
1-butanol, glacial acetic acid, pyridine, water
(3:3:1:1 v/v) (Abe and Samejima, 1975)

Chromatographic tanks were equilibrated with solvent overnight. Chromatographed plates were stained with a spray of ninhydrin (0.5%) in 1-butanol (Chromalay Ninhydrin Spray, May and Baker Ltd.). These were then heated at 100°C for 10 minutes.

2.10 Acid Hydrolysis

Dried samples were dissolved in concentrated AR-grade HCl (25 ml) and refluxed for 24 hours. The hydrolysate was evaporated to dryness on a rotary evaporator. Distilled water (5 ml each time) was added and the product evaporated to dryness again. This was repeated 3 times to remove remaining traces of HCl. Finally, the acid free hydrolysate was dissolved in distilled water and a sample removed for thin-layer

chromatography.

2.11 Cationic Electrophoresis

The discontinuous method of Reisfeld et al. (1962) was used for electrophoresis of basic proteins. Some modifications are described below.

Gel slabs were used instead of tubes. Two different templates were used in making the gel slabs.

(a) Two thin, chromic acid-cleaned glass plates (8x8 cm) separated on two sides by glass strips 3 mm thick were glued with PVA glue. The template was sealed with a strip of plasticine along the bottom edge. Acrylamide solutions were then pipetted into the template and allowed to polymerise. The plasticine was removed after polymerisation had occurred.

(b) Gels were also prepared using the apparatus supplied with the LKB 2001 vertical electrophoresis unit. Two chromic acid-cleaned glass plates (16x18 cm), were separated by two 1.5 mm thick spacers, and clamped together. This was then clamped in the gel casting stand which sealed the bottom edge. After polymerisation the glass plates were removed from the casting stand and clamped in the LKB vertical electrophoresis unit.

Two polyacrylamide gel zones were used:

zone 1, a 15% acrylamide small-pore gel in which electrophoretic separation is accomplished.

zone 2, a 3.3% acrylamide large-pore gel in which electrophoretic concentration takes place.

The unpolymerised gel mixture, zone 1, was pipetted into the template to the required level and overlaid with distilled water. After zone 1 had polymerised, the remaining surface liquid was washed off with a small quantity of unpolymerised zone 2 gel and the mould filled with this mixture. After polymerisation, the unpolymerised surface of the zone 2 gel was washed with tray buffer.

Stock solutions were stored in brown glass bottles at 4°C for several weeks. Stock solution A (60% acrylamide) was replaced with 30% acrylamide. Therefore in mixtures twice the volume specified by Reisfeld et al. (1962) for solution A was required. The extra volume was added in place of water in the given volumes.

Acrylic acid was removed from the acrylamide solutions by running these through a DEAE Sephadex A 25 anion exchanger which had been equilibrated with distilled water and packed in a Pharmacia chromatographic tube with a bed volume of 10 ml.

Protein samples were mixed with a drop of glycerol and applied to wells on the top of the large pore gel (generated by insertion of a Gradipore plastic applicator strip). When using the LKB template, the protein samples were applied to wells formed by insertion of a comb during the polymerisation of the large pore gel. Methyl green was used as a tracking dye.

Electrophoresis was carried out in a Gradipore electrophoresis tank for template (a), or in a LKB 2001 vertical electrophoresis unit for template (b), at a constant current of 40 mA for approximately 2 hours.

2.12 SDS Electrophoresis

Two different methods of SDS electrophoresis were used.

(1) The discontinuous method of Laemmli (1970) was routinely carried out. Gel slabs were used rather than rods, and these were prepared as described for cationic electrophoresis. Separating gels containing 15% acrylamide were used in preference to those recommended by Laemmli (1970).

Dried protein was added to sample solvent (100 µl) in screw cap vials and placed in a boiling water bath for 2 minutes to dissociate the proteins. Glycerol (1 drop) was then added, and aliquots were loaded onto the gels.

Anionic electrophoresis was carried out in an electrophoresis chamber at a constant current of 40 mA until the tracking dye approached the lower edge of the separating gel.

(2) The continuous method given in the bulletin "SDS Molecular Weight Markers" published by Sigma Chemical Company, was occasionally used. One change was made: urea was omitted from the sample buffer.

2.13 Gel Stains

Three different staining methods were used.

(1) Protein Stain using Coomassie Brilliant Blue R. The following solution was used:

Coomassie Brilliant Blue R	1.37 g
Methanol	250 ml
Glacial Acetic Acid	50 ml
Distilled Water	250 ml

Gels were stained for approximately 2 hours followed by destaining in 35% ethanol until a clear background was attained.

(2) Amido Black 10B. This was used as a suitable method of staining spermine, which is very poorly visualised by Coomassie Blue (Briggs, 1983).

Amido Black 10B	1 g
Glacial Acetic Acid	70 ml
Distilled Water	930 ml

The staining solution was heated to near boiling immediately before use (Ritchie et al., 1966). After staining 5 hours, gels were destained in a 7% acetic acid solution.

(3) The Silver Staining method as described by Merril et al. (1981) and the Bio-Rad bulletin 1089 (1982).

Step 1: Gels were fixed in 40% methanol/10% acetic acid overnight

Step 2: Rinsed twice for 30 minutes in 10% ethanol/5% acetic acid

Step 3: Soaked in oxidiser for 10 minutes (0.0034 mol/l potassium dichromate in 0.0032 mol/l nitric acid

Step 4: Rinsed 3 times (10 minutes each time) in distilled water

Step 5: Soaked in 0.012 mol/l Silver nitrate for 30 minutes under bright light

Step 6: Rinsed for 1 minute in distilled water

Step 7: Soaked in developer (0.28 mol/l sodium bicarbonate and 0.5 ml formalin/l) for 1 minute, replaced and soaked for a further 2 minutes. The developer was replaced thereafter whenever it turned yellow-brown, until the gel was fully developed.

Step 8: Development stopped in 5% acetic acid for 5 minutes

Step 9: Washed and stored in distilled water

2.14 Isoelectric Focusing

Isoelectric focusing was carried out using a Pharmacia Flat Bed Electrophoresis Apparatus (FBE 3000) and a 2,000 V constant wattage power supply (Isco Model 494).

Focusing was performed using LKB Ampholine PAGplates (9x11 cm) with a pH gradient of 3.5-9.5, and Servalt Pre-cotes (12.5x12.5 mm) with a pH gradient of 3-10.

The procedure used for setting up, running, fixing, staining and destaining the gels were as given in the booklets supplied with the gels.

Lysozyme, cytochrome c, myoglobin (Sigma Chemical Company) and the Protein Test Mixture 9 (Serva) were applied as standards and to follow the course of focusing. Isoelectric focusing was run at 4W with a limit of 2000 V and 20 mA for 2-3 hours when using the LKB PAGplates. The Serva Pre-cotes were run at 2.25 W with a limit of 1200 V and 5 mA for 2-3 hours.

After completion the pH gradient across the gel (LKB PAGplates) was measured with a surface electrode. The Serva Pre-cotes were too thin for an accurate surface reading so the pH gradient was determined from the standards in the Protein Test Mixture 9.

2.15 Protein Standards

The following protein standards obtained from Sigma Chemical Company were used in electrophoresis and isoelectric focusing.

lysozyme (type I from chicken egg white)

cytochrome c (type III from horse heart)

myoglobin (type I from horse skeletal muscle)

trypsinogen (type I from bovine pancreas)

ovalbumin (type V from egg white)

albumin (type V from bovine serum)

carbonic anhydrase (from bovine erythrocytes)

MW-SDS-70L (molecular weight standards)

Protein Test Mixture 9 (Serva) and molecular weight markers for SDS electrophoresis (BDH) were also used.

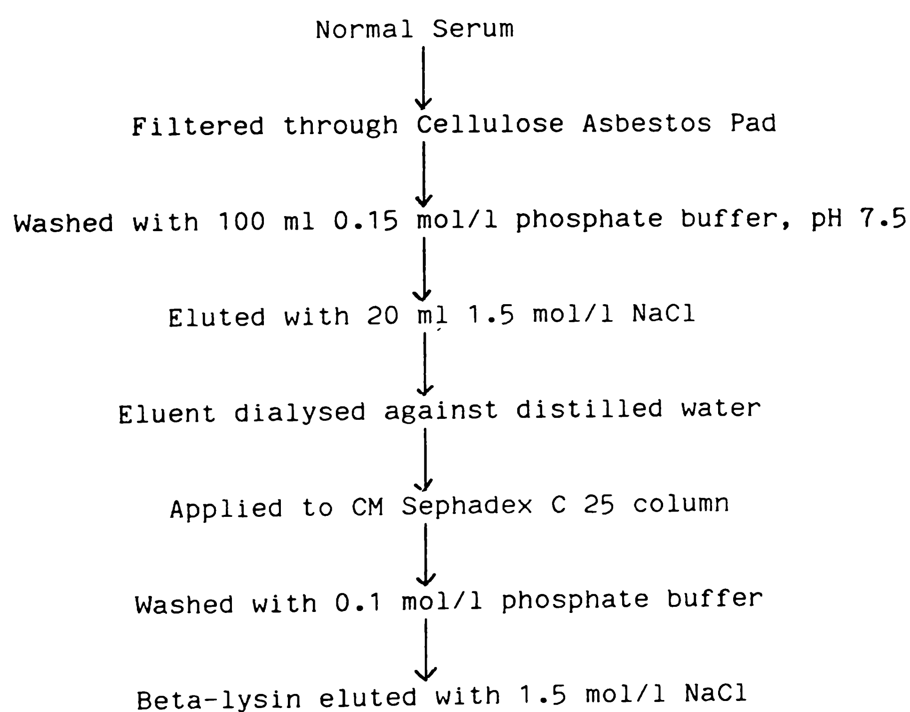
CHAPTER THREE

BETA-LYSIN

3.1 Introduction

The substance found to be responsible for the lethal effect of normal serum on a variety of Gram positive bacteria was named beta-lysin by Pettersson (1924). Donaldson and Marcus (1958) later demonstrated that blood coagulation mechanisms exert a mediating effect on the serum bactericidal activity, and Hirsch (1960b) established that beta-lysin originated from the blood platelets.

Donaldson and coworkers (1964) reported a purification procedure which effectively removes the beta-lysin from rabbit serum. This procedure was further modified by Johnson and Donaldson in 1968. The purification procedure is as follows:



Beta-lysin was also found to be present in the serum of rats, horses, humans and cows, although at lower concentrations than in rabbits (Donaldson and Tew, 1977)

3.2 Properties of Beta-lysin

The serum beta-lysins are cationic proteins with molecular weights of approximately 6 000 (Johnson and Donaldson, 1968). They are heat stable, losing little activity after incubation at 95°C for 30 minutes. Purified preparations of platelet beta-lysin have an ultraviolet absorption spectrum typical of proteins, and also show a positive protein reaction with the Lowry and Biuret protein detection reagents. The bactericidal activity is destroyed by proteolytic enzymes.

In normal serum, beta-lysin appeared to be attached to other serum protein fractions after either ammonium sulphate precipitation or Sephadex gel filtration chromatography of the serum (Donaldson and Tew, 1977). It was suggested that this may have been a result of the highly reactive cationic nature of the peptide.

As there was no published evidence that beta-lysin had been purified to homogeneity it was thought that it may have been isolated as an aggregated complex possibly containing spermine. It was known that beta-lysin was a compound of low molecular weight, very thermostable, with little known about its composition. There were no details on its amino acid content or isoelectric point, nor any electrophoretic proof of homogeneity. The molecular weight of 6 000 has been estimated by gel filtration chromatography on Sephadex G 200 although this value is well below the separation range of the gel. The limited characterisation of beta-lysin prompted this more detailed study.

3.3 Isolation of Beta-lysin from Bovine Blood

Bovine blood was used as the source of beta-lysin as it was readily obtainable from a local abattoir in large quantities.

Several litres of bovine blood were collected in a bucket containing an anticoagulant (50 ml of 4% sodium oxalate solution per litre of blood). The blood was centrifuged at 5 000 g for 20 minutes to remove most of the red blood cells. The supernatant was allowed to coagulate at room temperature after the addition of calcium chloride (2 g per litre of blood). The fibrin clot was removed and the remaining solution was either stored in 200 ml quantities at -20°C or purified by the following procedure:

200 ml of bovine serum was vacuum filtered through a cellulose asbestos pad. The filter pad was washed with 0.15 mol/l phosphate buffer, pH 7.5, then eluted with 20 ml of 1.5 mol/l NaCl which removed the beta-lysin fraction. The eluate was dialysed against 5 litres of distilled water for 24 hours to remove the salt. The dialysed beta-lysin solution was assayed for antibacterial activity on agarose plates seeded with Bacillus subtilis and the Bacillus sp. A low level of activity was detected, and it appeared that the Bacillus sp. was the more sensitive to beta-lysin.

Several preparations as described above were pooled and run onto a column of CM Sephadex C 25 equilibrated with 0.01 mol/l phosphate buffer, pH 7.5. The column was then eluted with 0.1 mol/l phosphate buffer, pH 7.5, followed by a final wash with 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer, pH 7.5. All fractions were assayed for antibacterial activity. The results are shown in Fig. 3-1. Only the fractions eluted with the 0.15 mol/l phosphate buffer pH 7.5 exhibited

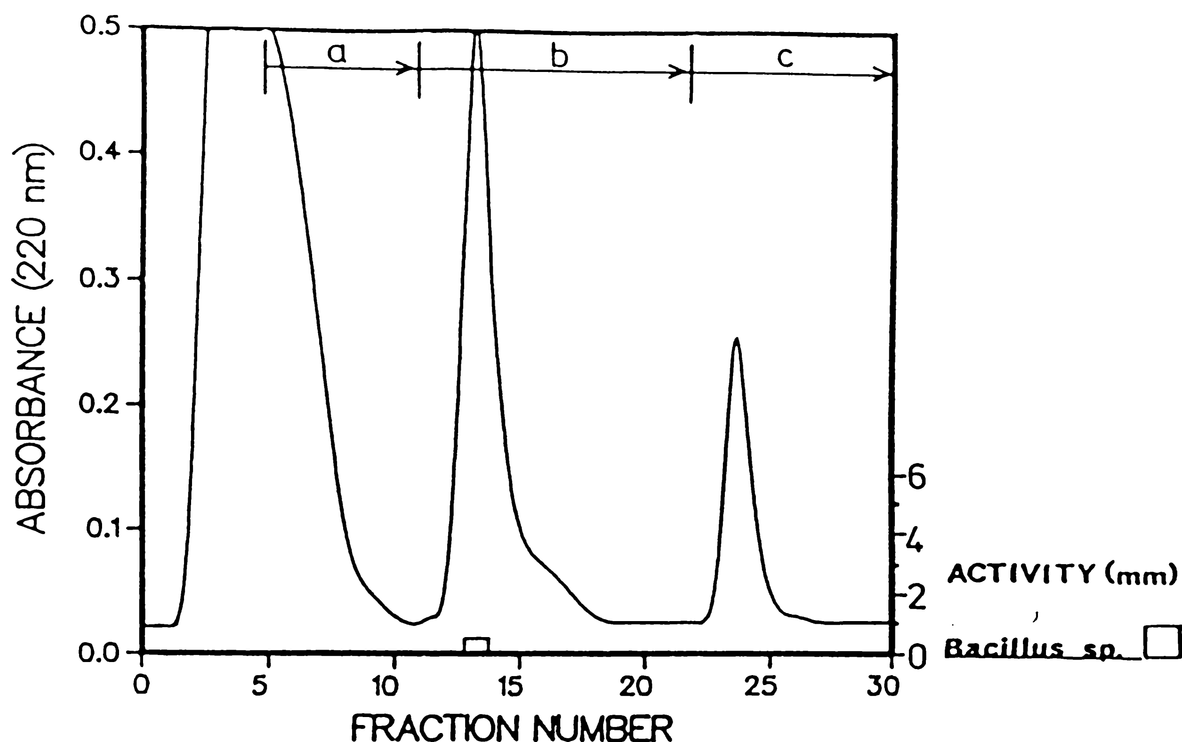


Fig. 3-1: Ion Exchange Chromatography of Crude Beta-lysin (Material Removed from the Cellulose Asbestos Filter Pad)
Gel: CM Sephadex C 25
Buffer: 0.01 mol/l phosphate, pH 7.5
Sample: The fraction eluted from the cellulose asbestos filter pad, desalted
Elution Buffers: (a) 0.01 mol/l phosphate buffer, pH 7.5;
(b) 0.15 mol/l phosphate buffer, pH 7.5;
(c) 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer, pH 7.5
Fraction Volume: 10 ml

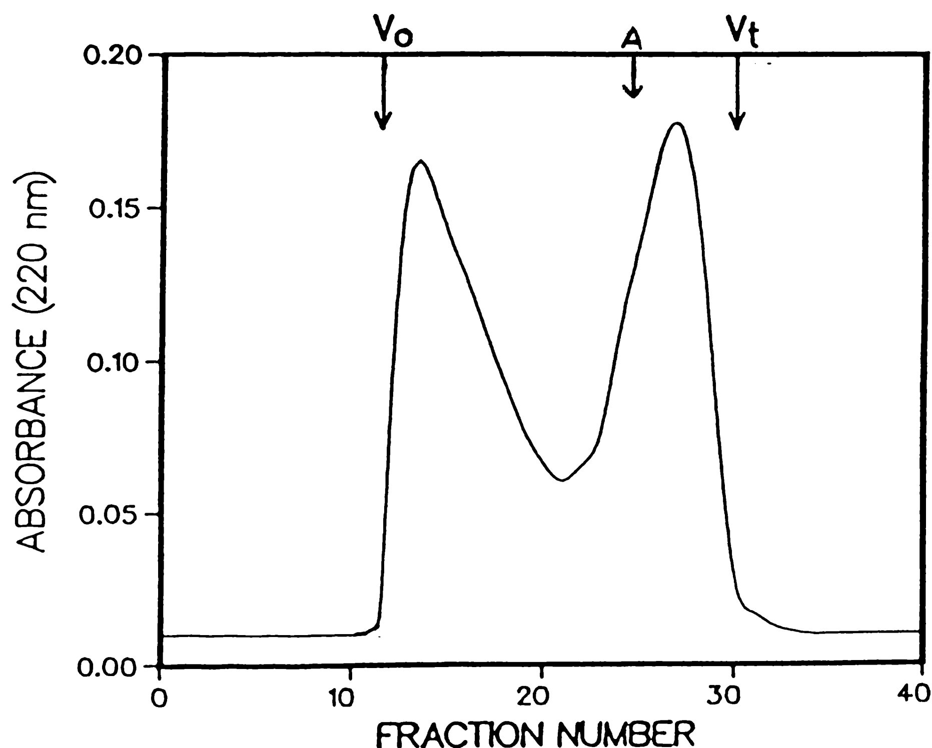


Fig. 3-2: Gel Filtration Chromatography of Fractions 23-25 from Fig. 3-1
Gel: Sephadex G 25 SF
Eluent: 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer, pH 7.5
Sample: Fractions 23-25 (Fig. 3-1), desalted, freeze-dried and re-dissolved in 5 ml of 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer, pH 7.5

antibacterial activity. To determine whether the high salt concentration may have been inhibiting any activity present from the material eluted by the 1.5 mol/l NaCl, fractions 12-18 and 23-25 were separately pooled and dialysed for 24 hours at 4°C against 5 litres of distilled water. Dialysis tubing with a nominal retention of 3 500 daltons was used to minimise the loss of low molecular weight proteins. After dialysis, the pooled fractions were assayed for antibacterial activity.

	Antibacterial Activity (mm)	
	Bacillus sp.	B. subtilis
Fractions 12-18	0.5	0.5
Fractions 23-25	3.0	3.0

Fractions 23-25 displayed strong activity, whereas fractions 12-18 were only slightly active. Fractions 23-25 represents beta-lysin as purified by Donaldson and coworkers (1968). The activity removed from the ion exchange column with 0.15 mol/l phosphate buffer (fractions 12-18) may have been beta-lysin bound to other proteins, which allowed it to be washed off by the lower ionic strength buffer, or may have been another antibacterial substance. This fraction was not studied further as the investigation only concerned beta-lysin as it was purified by the authors.

The purified beta-lysin fractions were chromatographed through a column of Sephadex G 25 to estimate their molecular weights. The separating range of Sephadex G 25 is approximately 1 500 to 5 000 daltons, making it a more accurate means of estimating the molecular weight than Sephadex G 200 as used by Johnson and Donaldson (1968). Fractions 23-25 were freeze-dried and redissolved in 5 ml of 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer, pH 7.5. This was chromatographed on a column of Sephadex G 25 eluted with 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer, pH 7.5. The results are shown in Fig. 3-2. Two large peaks (fractions 12-21 and 22-32) were eluted from the column indicating the heterogeneity of the beta-lysin sample. The column was previously calibrated with insulin (approximately 5 700 daltons) and insulin chain A (approximately 2 500 daltons) standards so as to estimate the molecular weight of the beta-lysin fraction. Insulin was eluted on the void volume of the column and insulin chain A was eluted as marked on Fig. 3-2. The molecular weights of the two peaks were estimated to be greater than 5 000 for fractions 12-21 (as it was eluted on the void volume) and approximately 2 000 for fractions 22-32. Fractions 12-21 and 22-32 were separately pooled and dialysed against 5 litres of distilled water for 24 hours at 4°C using dialysis tubing with a nominal retention of 3 500 daltons. After the salt had been removed the pooled fractions were assayed for antibacterial activity.

	Antibacterial Activity (mm)	
	Bacillus sp.	B. subtilis
fractions 12-21	1	1
fractions 22-32	2	2

Both pools of fractions exhibited antibacterial activity although fractions 22-32 were more active. The retention of the activity of fractions 22-32 by the dialysis tubing indicated a molecular weight of greater than 3 500 and not 2 000 as estimated from gel filtration chromatography. Samples (1 ml) were taken from each pool and freeze-dried. They were dissolved in sample buffer and separated by discontinuous cationic electrophoresis (see Fig. 3-3). Fractions 12-21 and 22-32 both showed up as single bands with the same mobility as spermine. However it has been previously noted that small cationic proteins have similar migration rates to spermine when using this electrophoretic method (Briggs, 1983). Fractions 12-21 may have contained spermine bound on to higher molecular weight material, and fractions 22-32 may have been spermine co-eluting with other protein material.

Fractions 12-21 and 22-32 were examined for their spermine content on thin-layer chromatography. Fractions 12-21 and 22-32 were freeze-dried and the product (4.5 mg from fractions 12-21 and 4.3 mg from fractions 22-32) were hydrolysed in concentrated HCl for 24 hours. The hydrolysates were rotary evaporated to dryness and three times 5 ml

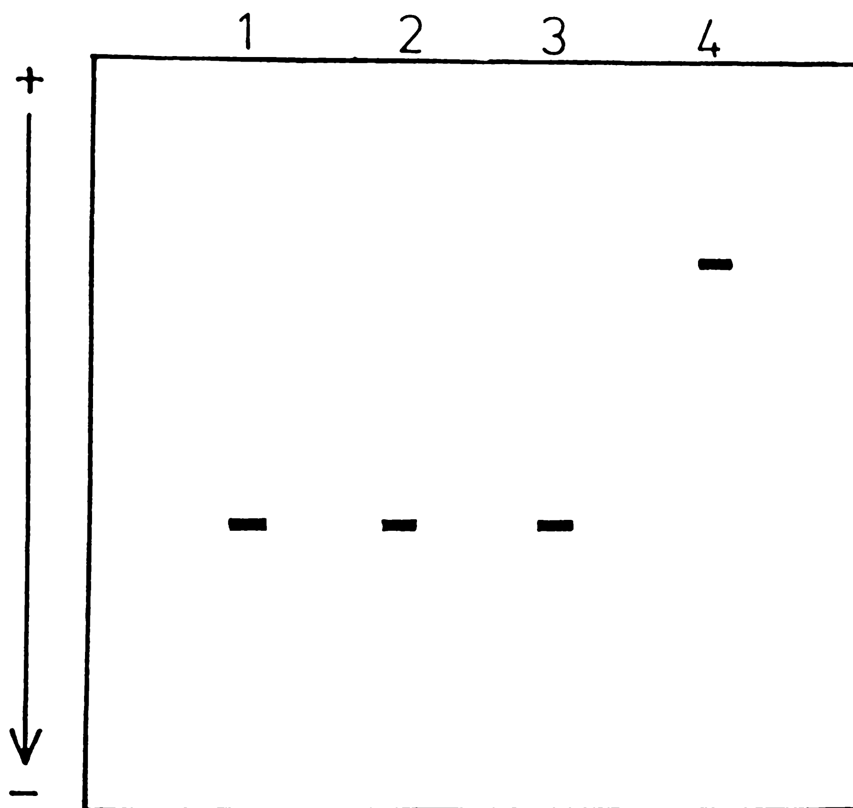


Fig. 3-3: Cationic Electrophoresis
1 - spermine standard
2 - fractions 12-21 from Fig. 3-2
3 - fractions 22-32 from Fig. 3-2
4 - lysozyme standard

of distilled water was added and taken to dryness to remove all traces of the HCl. The products were re-dissolved in 50 µl of distilled water and 2 µl samples were chromatographed by thin-layer chromatography using silica gel G. The ninhydrin-stained plate showed that fractions 12-21 and 22-32 contained no spermine (see Fig. 3-4), even when grossly overloaded (10 µl) samples were applied. It is possible that spermine may have been present but below the level of detection. However as the concentration of the sample (90 mg/ml) is very high, and it is possible to detect spermine easily at concentrations of 1 mg/ml, then even if present the concentration of spermine would make a negligible contribution to the total antibacterial activity.

Thus it was concluded that beta-lysin is in fact a cationic protein and does not contain spermine. As little previous characterisation had been carried out on beta-lysin, it was examined more throughly.

3.4 Purification of Bovine Beta-lysin

Bovine blood was purified as previously outlined to obtained beta-lysin as described by Johnson and Donaldson (1968). The active fractions eluted from the column of CM Sephadex C 25 with 1.5 mol/l NaCl (the same as fractions 23-25, Fig. 3-1) were pooled and dialysed to remove the salt. They were assayed and found to have antibacterial activity. The pooled fractions were freeze-dried. The product was re-dissolved in 0.075 mol/l phosphate buffer pH 7.5 containing 1.5 mol/l NaCl and chromatographed on a column of Sephadex G 25 eluted with the same buffer. The results are shown in Fig. 3-5. The elution profile was different from that previously obtained for a similar run (shown in Fig. 3-2). After dialysis of the pooled peaks only the peak at the void volume (fractions 12-15) exhibited antibacterial activity. Fractions

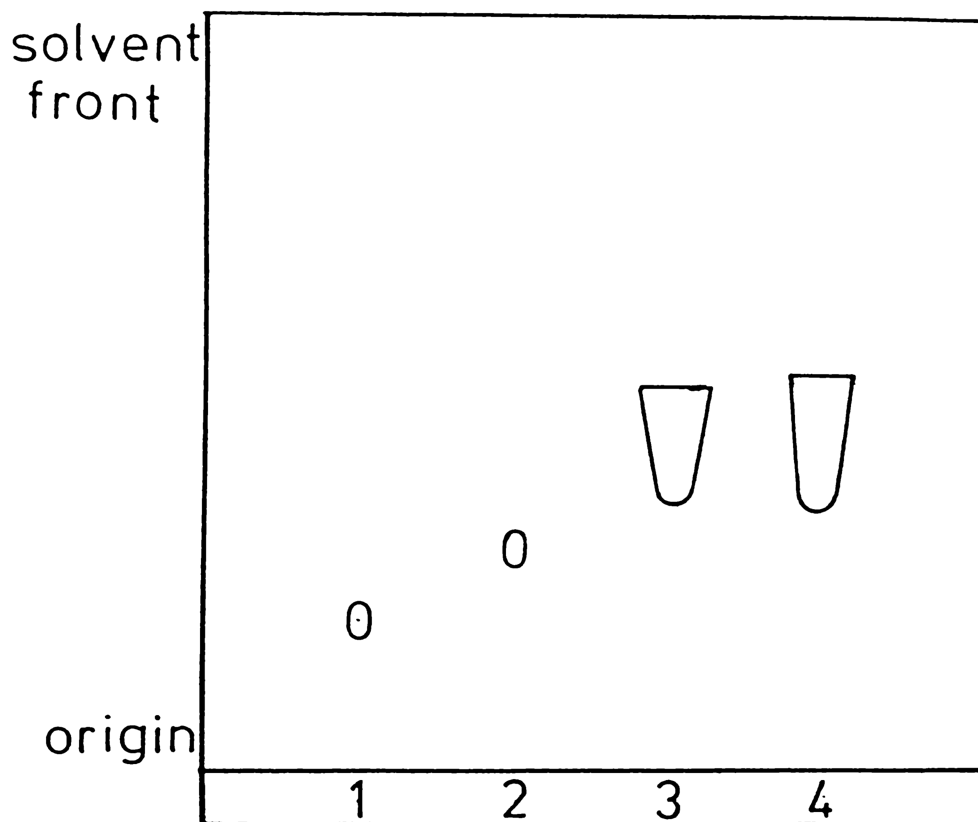


Fig. 3-4: Thin-layer Chromatography
1 - spermine standard
2 - spermidine standard
3 - fractions 12-21 from Fig. 3-2
4 - fractions 22-32 from Fig. 3-2

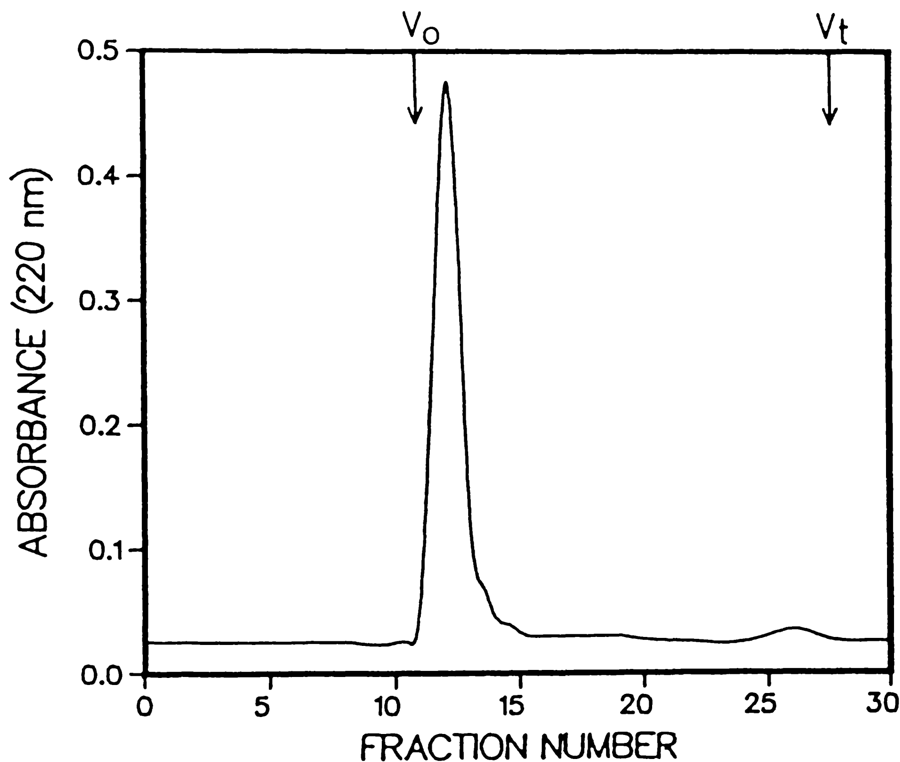


Fig. 3-5: Gel Filtration Chromatography of Fractions 23-25 from Fig. 3-1
Gel: Sephadex G 25 SF
Eluent: 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer, pH 7.5
Sample: Fractions 23-25 (Fig. 3-1), desalted, freeze-dried and re-dissolved in 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer, pH 7.5
Fraction Volume: 10 ml

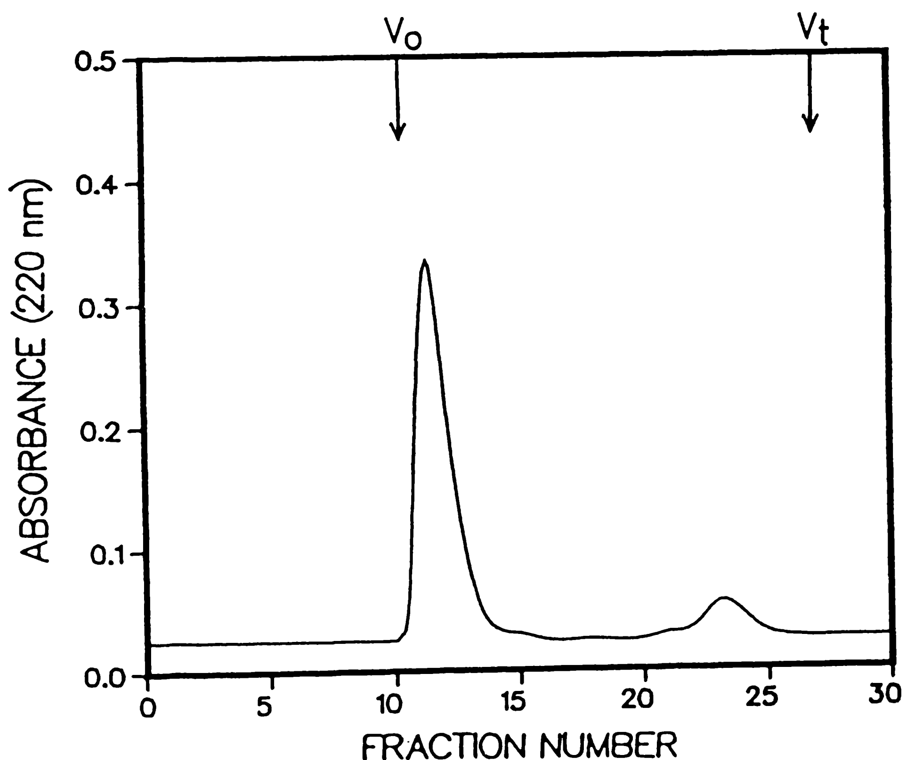


Fig. 3-6: Gel Filtration Chromatography of Fractions 12-15 from Fig. 3-5
Gel: Sephadex G 50 SF
Eluent: 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer, pH 7.5
Sample: Fractions 12-15 (Fig. 3-5), desalted, freeze-dried and re-dissolved in 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer, pH 7.5

12-15 were freeze-dried and redissolved in 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer pH 7.5. This sample was then chromatographed on a column of Sephadex G 50 eluted with 1.5 mol/l NaCl in 0.075 mol/l phosphate buffer pH 7.5. The results are shown in Fig. 3-6. Two peaks were eluted from the column; fractions 11-14 and 23-25. These fractions were separately pooled, dialysed and assayed for antibacterial activity. Only fractions 23-25 were active.

To examine the degree of homogeneity of the beta-lysin fractions obtained from the purification procedure, samples were examined by SDS electrophoresis. Salt-free samples of fractions 12-15 (Fig. 3-5) and fractions 11-14 and 23-25 (Fig. 3-6) were freeze-dried. They were dissolved in SDS sample buffer and run on SDS electrophoresis as shown in Fig. 3-7. The beta-lysin (fractions 23-25 from Fig. 3-6) appeared as a major band which migrated further than the lysozyme and cytochrome c standards. The beta-lysin fraction was not entirely homogeneous, as 2 other bands with slower migration rates were present. However, these bands were also present in the pooled fractions 11-14 (Fig. 3-6) which had no antibacterial activity, therefore they were probably inactive contaminants.

Samples were also separated by SDS electrophoresis with BDH molecular weight markers (molecular weight range 2 512 - 16 949) to estimate the molecular weight of beta-lysin (Fig. 3-8). The logarithm of molecular weight was plotted against the R_f for the standards (Fig. 3-9). The molecular weight for beta-lysin was estimated from its R_f value to be 6 100.

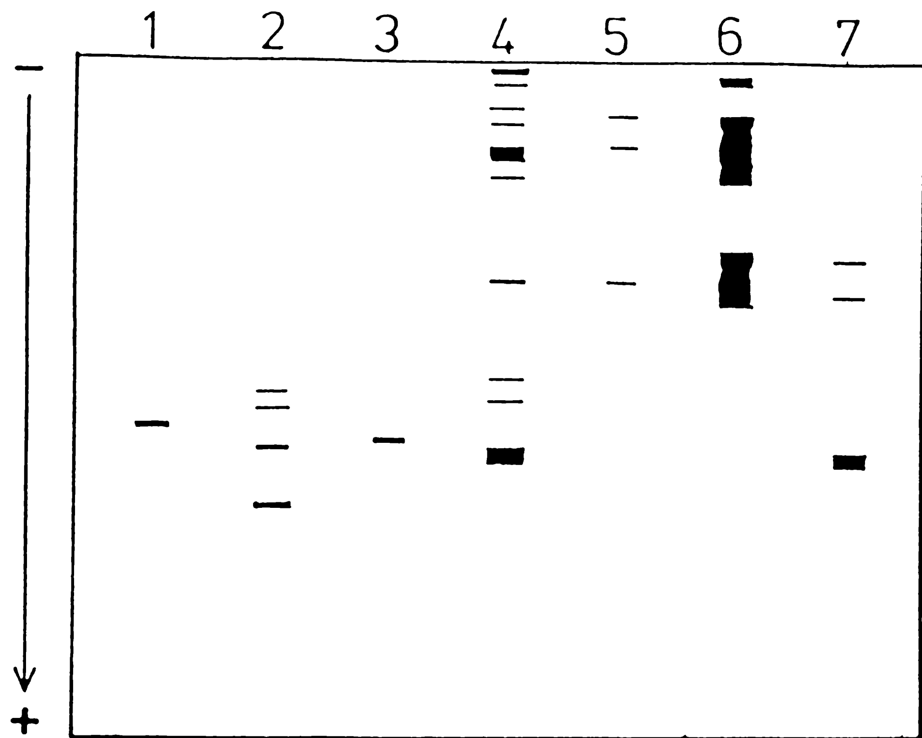


Fig. 3-7: SDS Electrophoresis
1 - lysozyme standard
2 - BDH molecular weight standards
3 - cytochrome c standard
4 - fractions 23-25 from Fig. 3-1
5 - fractions 25-28 from Fig. 3-5
6 - fractions 11-14 from Fig. 3-6
7 - fractions 23-25 from Fig. 3-6

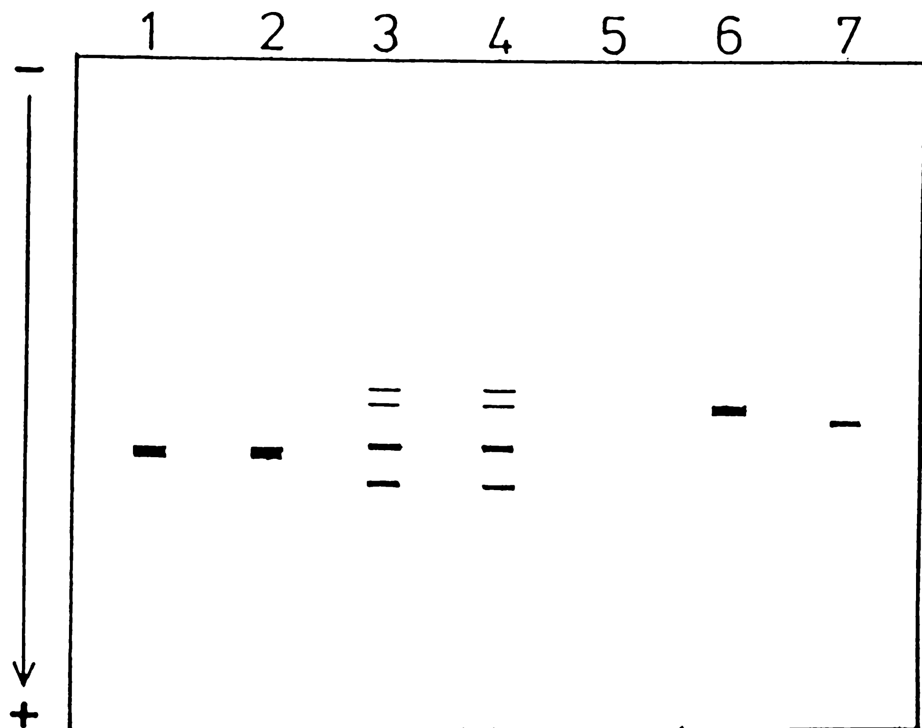


Fig. 3-8: SDS Electrophoresis
1 - beta-lysin
2 - beta-lysin
3 - BDH molecular weight standards
4 - BDH molecular weight standards
5 -
6 - cytochrome c standard
7 - lysozyme standard

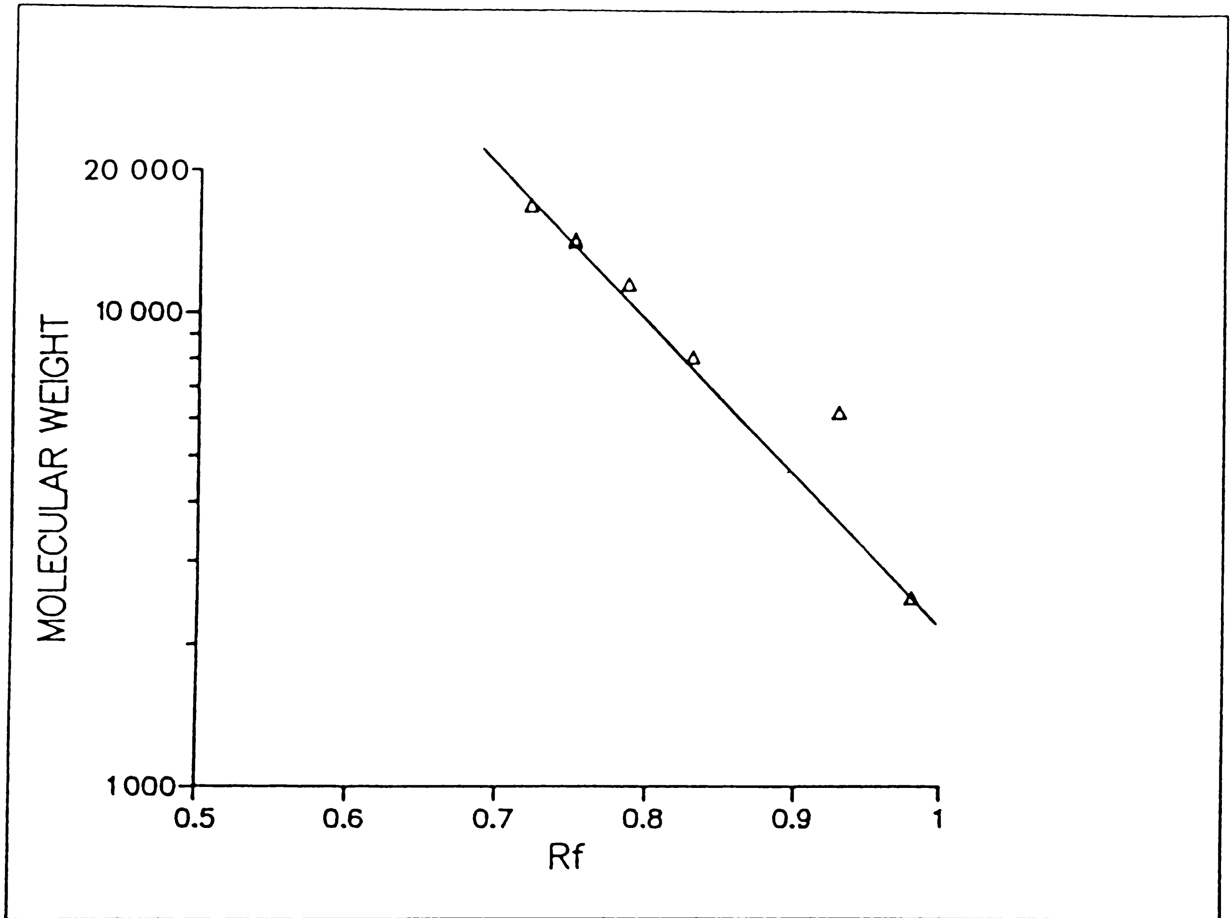


Fig. 3-9: Calibration Curve of Standards Run on SDS Electrophoresis

Attempts were also made to ascertain the isoelectric point of beta-lysin on LKB PAGplates with a focusing range from pH 3.5-9.5. However this proved unsuccessful as bands could not be stained. This was either the result of the low solubility of the beta-lysin or the protein may not have focused in the range of the gel owing to its highly cationic nature.

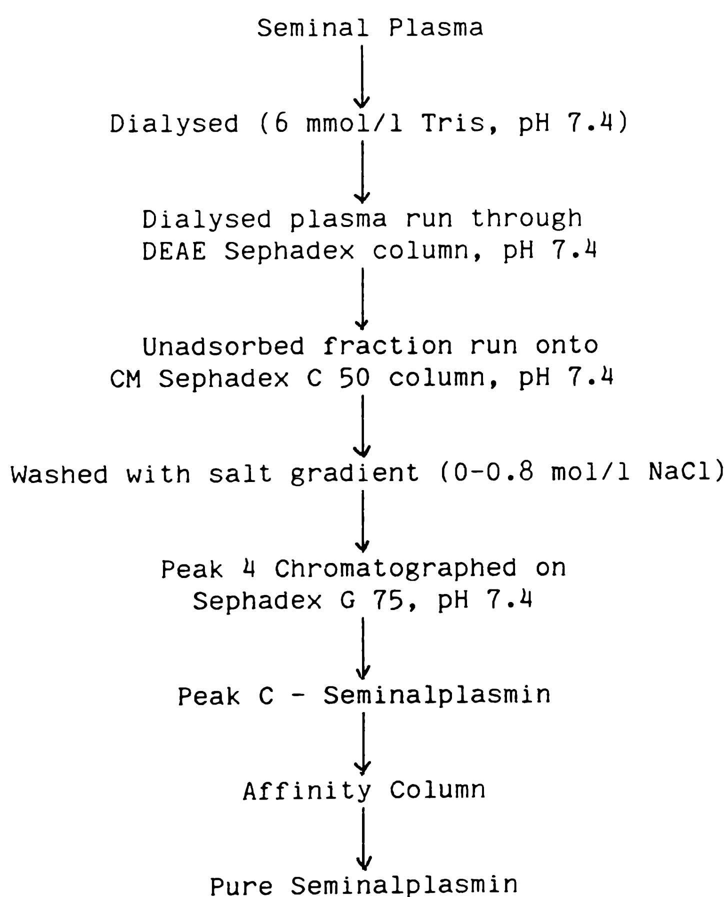
No further work on this topic was carried out after the publication by Carroll and Martinez (1981a,b,c) of a complete characterisation of rabbit serum beta-lysin which they named PC-III.

CHAPTER FOUR

SEMINALPLASMIN

4.1 Introduction

Reddy and Bhargava (1979) isolated and characterized an antibacterial protein from bovine seminal plasma. Isolation of the antibacterial protein (seminalplasmin) was achieved by the following procedure:-



4.2 Properties of Seminalplasmin

Seminalplasmin was reported to have a molecular weight of between 8 000 (by ultracentrifugation) and 19 800 (calculated from amino acid analysis), and an isoelectric point at pH 9.8. Seminalplasmin strongly inhibited the growth of Gram-positive and Gram-negative bacteria and

yeasts. It was also heat-stable, being relatively unaffected by heating at 90°C for 10 minutes.

As a result of the reported variation in molecular weight it was thought that seminalplasmin may not have been purified to homogeneity, and may have been isolated as an aggregated complex containing spermine.

4.3 Isolation of Seminalplasmin

In attempts to follow the published purification sequence of Reddy and Bhargava (1979), it was found that insufficient detail was provided to exactly duplicate the original procedure. The purification steps adopted are compared with those of Reddy and Bhargava (1979).

Bovine seminal plasma (200 ml) was collected from vasectomized bulls and dialysed against 5 litres of 6 mmol/l Tris buffer pH 7.4, at 4°C for 48 hours. During the dialysis a precipitate appeared which was not mentioned in the original publication. The precipitate was removed by centrifugation and on subsequent testing was found to have no antibacterial activity against E. coli and the Bacillus sp.

The dialysed seminal plasma was passed through a column of DEAE Sephadex A 25 equilibrated with 6 mmol/l Tris buffer, pH 7.4. The unadsorbed fraction from the DEAE Sephadex column was then chromatographed on a column of CM Sephadex C 25 equilibrated with 6 mmol/l Tris buffer, pH 7.4. The column was eluted with a linear salt gradient from 0 to 0.8 mol/l NaCl in 6 mmol/l tris buffer pH 7.4. The volume of the salt gradient was not specified by Reddy and Bhargava (1979) so a volume of 300 ml was used. The volume of the salt gradient may be critical as many more peaks were eluted from the column than reported in the published procedure (compare Fig. 4-1a with Fig. 4-1b).

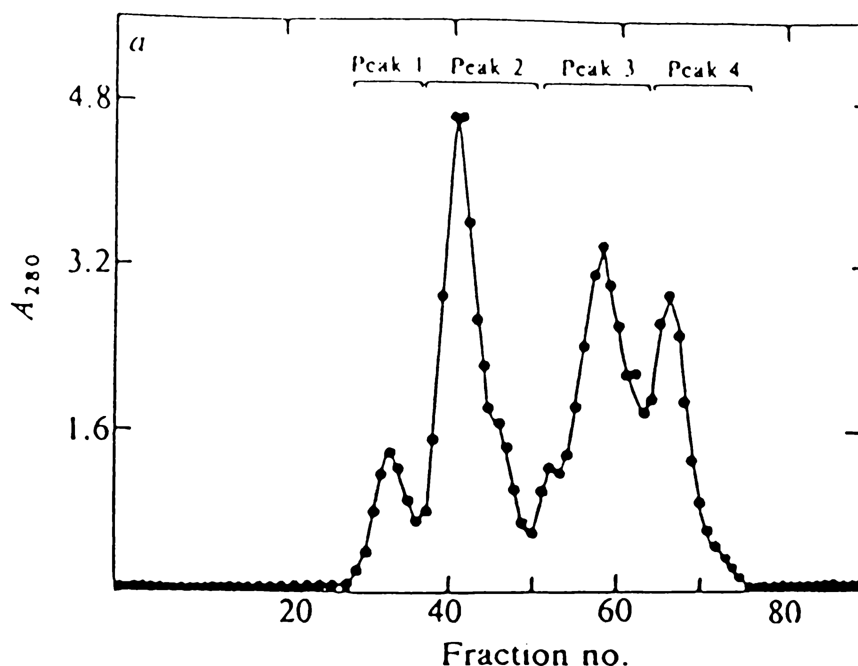


Fig. 4-1a: Ion Exchange Chromatography of the Unabsorbed Fraction from the Column of DEAE Sephadex - reprinted from Reddy and Bhargava (1979)
Gel: CM Sephadex C 50
Buffer: 6 mmol/l Tris, pH 7.4
Sample: Unabsorbed seminal plasma fraction eluted through the column of DEAE Sephadex
Elution Buffer: salt gradient from 0 to 0.8 mol/l NaCl in 6 mmol/l Tris buffer, pH 7.4
Fraction Volume: Unknown

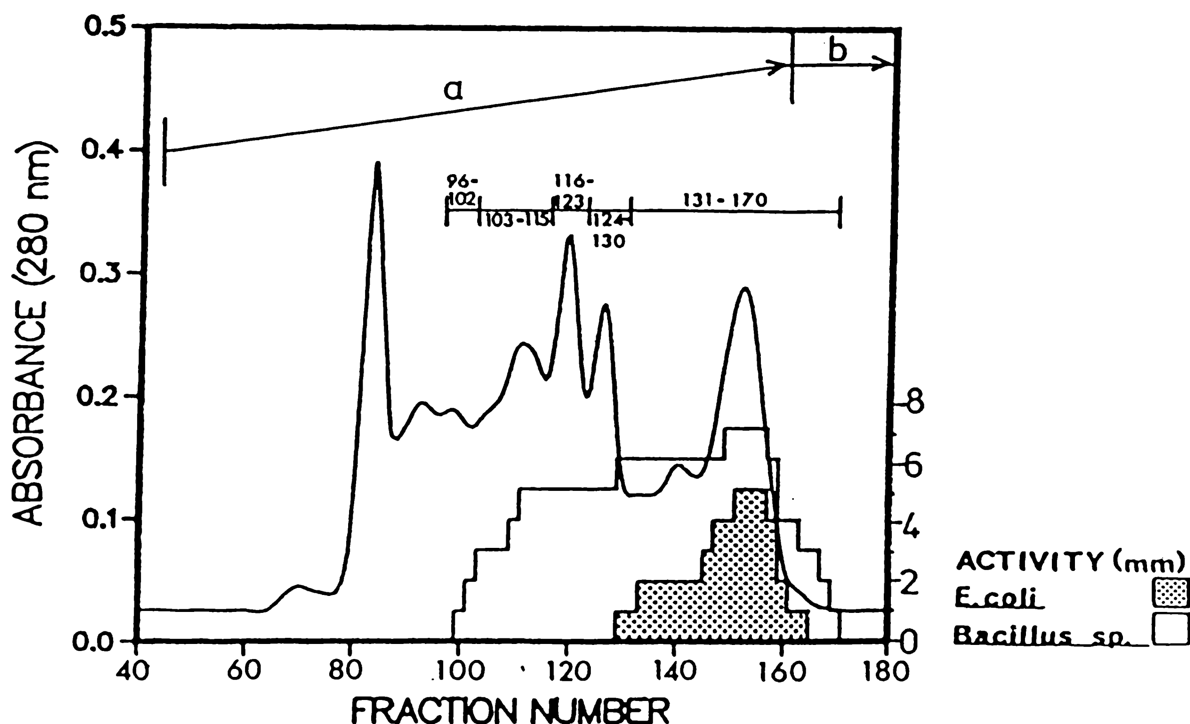


Fig. 4-1b: Ion Exchange Chromatography of the Unabsorbed Fraction of seminal plasma from the column of DEAE Sephadex
Gel: CM Sephadex C 50
Buffer: 6 mmol/l Tris buffer, pH 7.4
Sample: Unabsorbed seminal plasma fraction eluted through the column of DEAE Sephadex
Elution Buffers: (a) salt gradient from 0 to 0.8 mol/l NaCl in 6 mmol/l Tris buffer, pH 7.4; (b) 0.8 mol/l NaCl in 6 mmol/l Tris buffer, pH 7.4
Fraction Volume: 5 ml

Reddy and Bhargava found antibacterial activity against the *E. coli* test organisms largely in peak 4 and to a lesser extent in the tail of peak 3 (Fig. 4-1a). In the present work (Fig. 4-1b), the antibacterial activity against *E. coli* was associated with the last peak, fractions 131-170 (Fig. 4-1b). However antibacterial activity was also detected in fractions 96-170 when using the *Bacillus* species as a test organism. This suggests that another antibacterial protein is present which is not active against the *E. coli* test organism, or that the increased sensitivity of the *Bacillus* species for seminalplasmin has detected a large spread of activity due to complexes formed between seminalplasmin and other proteins.

The peaks in Fig. 4-1b were pooled as marked, dialysed against distilled water and freeze-dried. A sample from each of the freeze-dried pools was dissolved in SDS sample buffer (2 mg/ml) and separated by SDS electrophoresis to visualise the separation being achieved and the difference between the active fractions. The results are shown in Plate 4-1. Each of the pooled samples showed a large number of bands, many of which were common to all fractions. The most mobile band was the one most likely to correspond to seminalplasmin, according to the molecular weight estimation (Reddy and Bhargava, 1979) using the SDS electrophoretic method of Laemmli (1970). It is interesting that this band is common to all pooled fractions except fractions 116-123.

Samples (2 mg dissolved in 1 ml of water) of each of the pooled fractions were also separated by cationic discontinuous electrophoresis at pH 4.5 (Reisfeld, 1962). The stained gel (as shown in Plate 4-2) again showed a complex mixture of proteins present in each fraction. In fractions 96-102, 103-115 and 131-170 bands with migration rates

Plate 4-1: SDS Electrophoresis

1 - lysozyme standard

2 - cytochrome c standard

3 - trypsinogen standard

4 - fractions 96-102 from Fig. 4-1b

5 - fractions 103-115 from Fig. 4-1b

6 - fractions 116-123 from Fig. 4-1b

7 - fractions 124-130 from Fig. 4-1b

8 - fractions 131-170 from Fig. 4-1b

Plate 4-2: Cationic Electrophoresis

1 - lysozyme standard

2 - fractions 96-102 from Fig. 4-1b

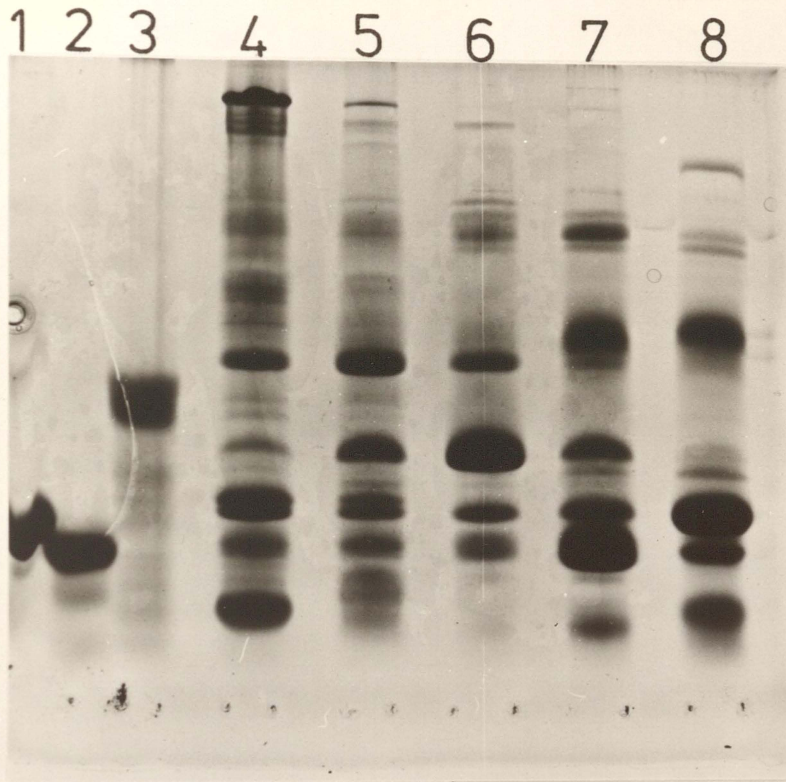
3 - fractions 103-115 from Fig. 4-1b

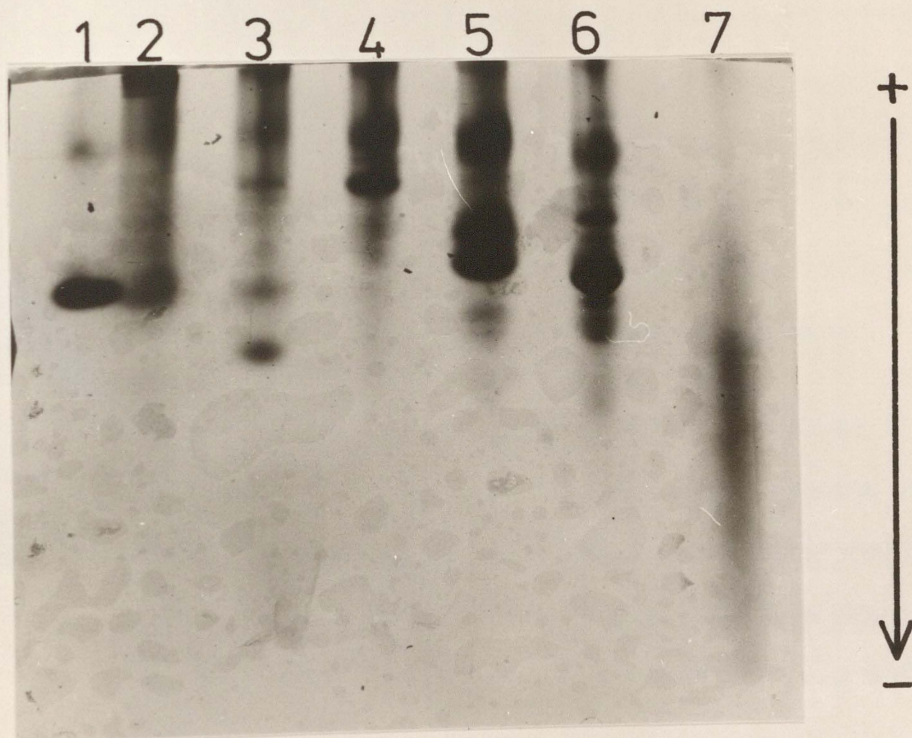
4 - fractions 116-123 from Fig. 4-1b

5 - fractions 124-130 from Fig. 4-1b

6 - fractions 131-170 from Fig. 4-1b

7 - not relevant


1 2 3 4 5 6 7 8
−
+


1 2 3 4 5 6 7
+
−

identical to that of the lysozyme standard were observed. It is possible, therefore, that lysozyme is responsible in part for the large spread of activity observed (Fig. 4-1b). It has been shown that lysozyme is present in seminal plasma and is eluted from a column of CM Sephadex C 25 with approximately 0.4 mol/l NaCl (Eschenbruch, 1980).

The freeze-dried fractions 131-170 from ion exchange chromatography, were re-dissolved in 6 mmol/l Tris buffer pH 7.4, and chromatographed on a column on Sephadex G 75 F eluted with 6 mmol/l Tris buffer pH 7.4. The results are shown in Fig. 4-2b. In the procedure published by Reddy and Bhargava (1979), gel filtration chromatography of peak 4 (Fig. 4-1a) on Sephadex G 75 resulted in an elution profile which gave 3 peaks, the last of which (fraction C) was the active seminalplasmin fraction (see Fig. 4-2a).

In the present procedure, 3 peaks were obtained as shown in Fig. 4-2b. The last two peaks (Fractions B and C) were active against the Bacillus species, while only Fraction C was active against E. coli. Fractions A, B and C were pooled separately, dialysed against distilled water and freeze-dried. Samples of the freeze-dried fractions (1 mg/ml) were re-suspended in 6 mmol/l Tris buffer, pH 7.4 and re-tested for antibacterial activity. Only fraction B was found to be active, against the Bacillus sp. but not against E. coli. However fraction C did not appear to be soluble which may account for the lack of activity.

Samples of fraction A, B and C (1 mg/ml) were dissolved in SDS sample buffer and incubated at 100°C for 2 minutes before being applied to SDS electrophoresis gels. Fraction C did not dissolve entirely, and was centrifuged prior to application. Electrophoresis was carried out by the method of Laemmli (1970). The results are shown in Plate 4-3.

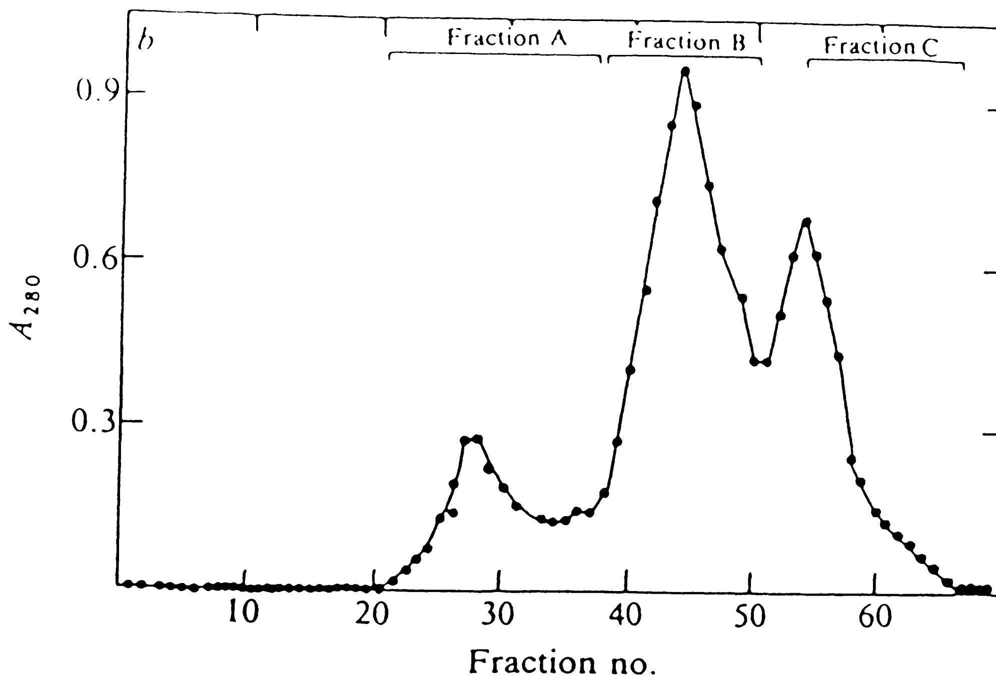


Fig. 4-2a: Gel Filtration Chromatography of Peak 4 from Fig. 4-1a - reprinted from Reddy and Bhargava (1979)
Gel: Sephadex G 75
Eluent: 6 mmol/l Tris buffer, pH 7.4
Sample: Peak 4 (Fig. 4-1a), desalted, freeze-dried and redissolved in 6 mmol/l Tris buffer, pH 7.4
Fraction Volume: Unknown

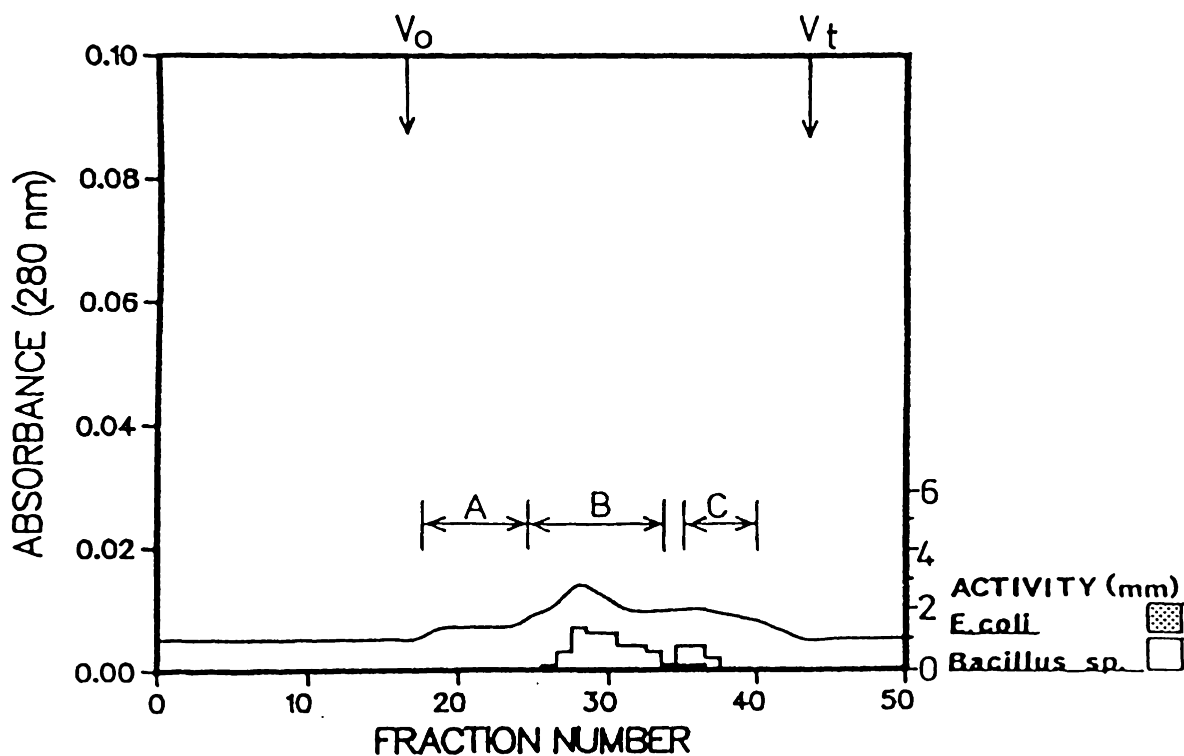


Fig. 4-2b: Gel Filtration Chromatography of Fractions 131-170 from Fig. 4-1b
Gel: Sephadex G 75 F
Eluent: 6 mmol/l Tris buffer, pH 7.4
Sample: Fractions 131-170 (Fig. 4-1b), desalted, freeze-dried and re-dissolved in 5 ml of 6 mmol/l Tris buffer, pH 7.4
Fraction Volume: 10 ml

Plate 4-3: SDS Electrophoresis

1 - cytochrome c standard

2 - lysozyme standard

3 - trypsinogen standard

4 - myoglobin standard

5 - Fraction A from Fig. 4-2b

6 - Fraction B from Fig. 4-2b

7 - Fraction C from Fig. 4-2b

Plate 4-4: Cationic Electrophoresis

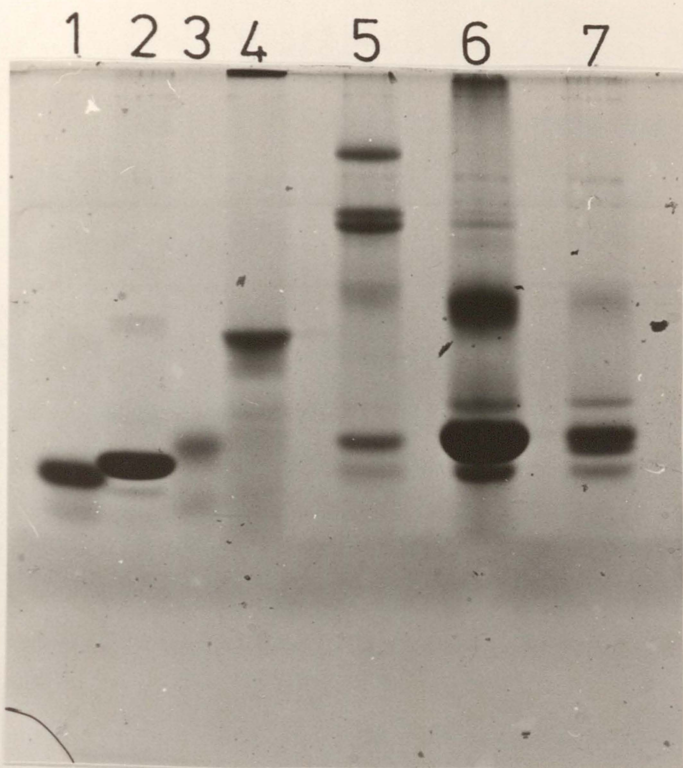
1 - fractions 131-170 from Fig. 4-1b

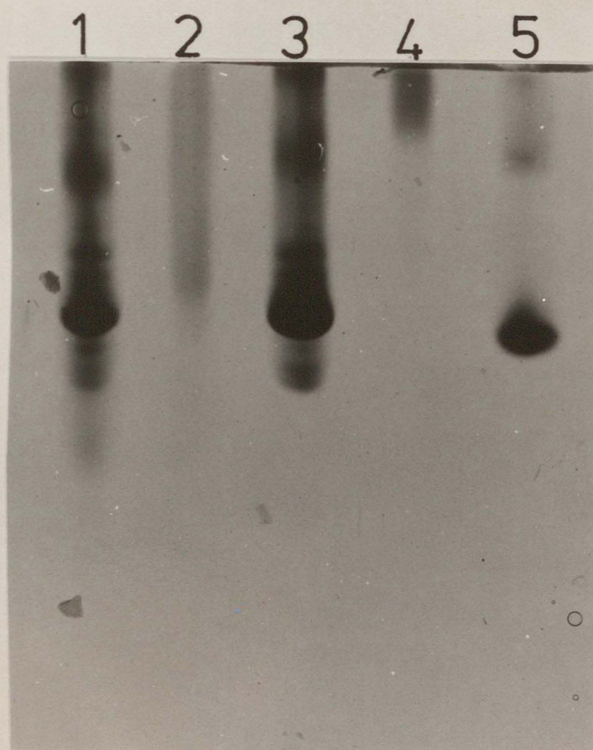
2 - Fraction A from Fig. 4-2b

3 - Fraction B from Fig. 4-2b

4 - Fraction C from Fig. 4-2b

5 - lysozyme standard


1 2 3 4 5 6 7
−
+


1 2 3 4 5
+
−

Fraction C (which should be pure seminalplasmin) contained several bands, similar to those in fraction B. This indicated that either seminalplasmin was present in both fractions, or was lost during the dialysis or solubilisation steps.

Attempts were also made to dissolve samples of fraction A, B and C in the sample buffer for cationic discontinuous electrophoresis. Although both fractions A and C appeared to be insoluble, the samples were electrophoresed after the precipitates had been removed. Fractions A and C did not run as bands but appeared as streaks in the gel (see Plate 4-4). This is probably due to the low solubility of these fractions. It was noted again that the lysozyme standard migrated the same distance as the heavily stained band in fraction B.

As it appeared that the seminalplasmin was lost during the purification sequence the affinity chromatography step was not carried out. Subsequent tests showed that fraction C was insoluble in distilled water, various concentrations of NaCl, ethanol, methanol, ether, acetone, petroleum spirit, guanidine HCl, and urea.

This procedure was repeated 3 times in an attempt to obtain seminalplasmin as described by Reddy and Bhargava (1979). However each time the end product was not homogeneous on SDS electrophoresis and was almost totally insoluble except in SDS sample buffer. Antibacterial activity could not be detected in a suspension of fraction C but this could have been due to the low solubility of this fraction.

4.4 Testing for the Presence of Spermine

Various fractions were tested for the presence of spermine. Samples were hydrolysed and subsequently run on thin-layer chromatography. Kido et al. (1980) successfully recovered spermidine from a covalent conjugate, 1008 B, extracted from Pseudomonas fluorescens, after refluxing for 13 hours in HCl (6 mol/l). Hettinger et al. (1968) also recovered spermidine from another covalent conjugate, edeine B which was extracted from Bacillus brevis and refluxed for 30 hours in HCl (6 mol/l). This type of procedure was used with the following fractions.

Samples (2 mg) from the freeze-dried fractions A, B and C (Fig. 4-2b) and fractions 131-170 (Fig. 4-1b) were each refluxed in 25 ml of 6 mol/l HCl for 24 hours. The hydrolysates were evaporated to dryness in a rotary evaporator. Distilled water (5 ml) was added and the product evaporated to dryness to remove the HCl. This was repeated three times.Finally, the hydrolysate was dissolved in 50 µl of distilled water and 2 µl samples were used for thin-layer chromatography (Fig. 4-3). Spermine and spermidine were run as standards.

After spraying with ninhydrin the silica gel plate was incubated at 110°C for 10 minutes. Heavy staining was detected in the upper and middle regions of the gel, which was attributed to the amino acids present. However no spermine or spermidine were detected in these samples at the applied concentrations. Polyamines could not be detected even when applying grossly overloaded samples (10 µl).

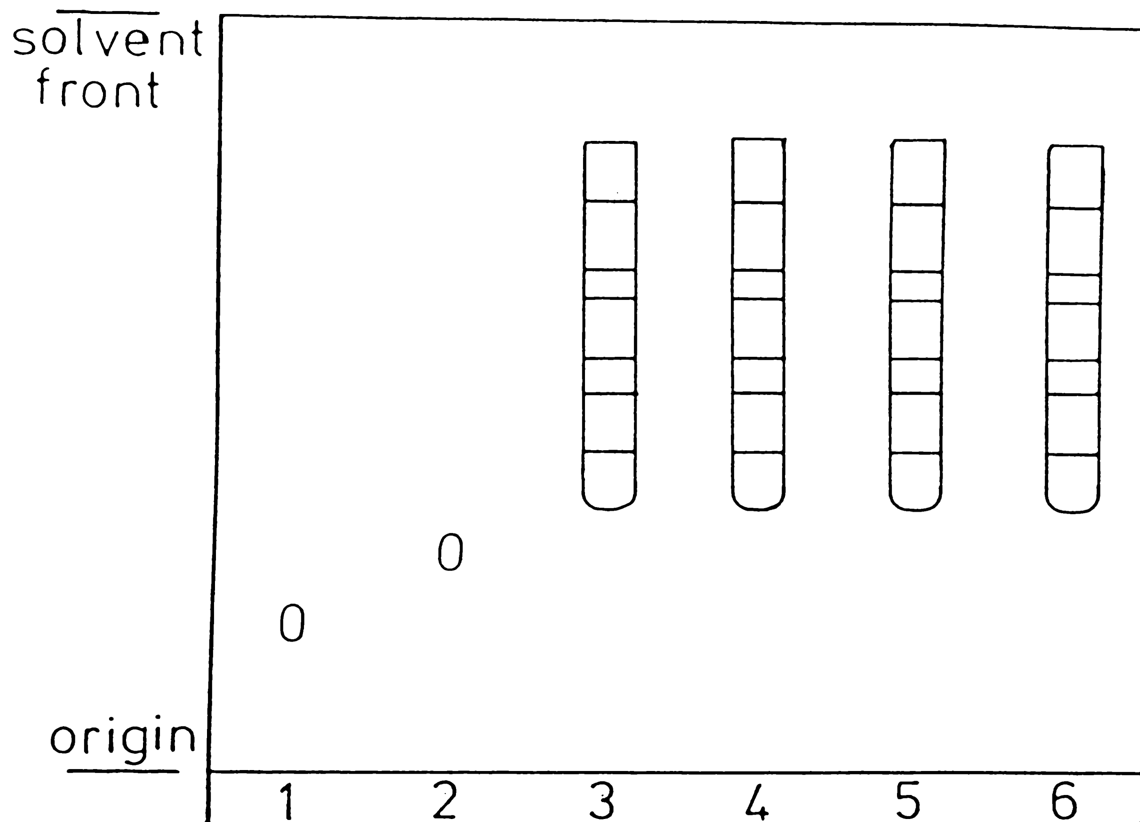


Fig. 4-3: Thin-layer Chromatography
1 - spermine standard
2 - spermidine standard
3 - Fraction A from Fig. 4-2b
4 - Fraction B from Fig. 4-2b
5 - Fraction C from Fig. 4-2b
6 - Fractions 131-170 from Fig. 4-1b

Spermine at a concentration of no less than 0.5 mg/ml was detectable after thin-layer chromatography when applied as a 2 µl sample. The maximum level that could therefore be present in the hydrolysed fraction without being detected would be less than 1.25%.

4.5 Further purification of Seminalplasmin

It was concluded that the purification step most likely to result in the loss of seminalplasmin would be the dialysis. Although the minimum molecular weight of seminalplasmin was 19 800 when calculated from the amino acid composition (Reddy and Bhargava, 1979), its molecular structure might allow it to escape from the dialysis tubing which has a nominal molecular weight retention of 10 000. Therefore, in order to eliminate the use of a dialysis step an alternative purification procedure was adopted. Different eluents were also used during gel filtration chromatography in an attempt to obtain more satisfactory separation of the antibacterial proteins present.

Material that had been purified on the column of CM Sephadex C 50 (fractions 131-170 in Fig. 4-1b) and which had been dialysed and freeze-dried but was still active against the E. coli test organism (suggesting that it still contained seminalplasmin) was purified further. The freeze-dried material was re-dissolved in 0.02 mol/l HCl, pH 1.7 and chromatographed on a column of Sephadex G 50 F eluted with 0.02 mol/l HCl. The results are shown in Fig. 4-4. Two distinct regions of activity were resolved: a high molecular weight region (fractions 10-16) which was predominantly active against the Bacillus sp. and a low molecular weight region (fractions 17-21) which was active against E. coli as well.

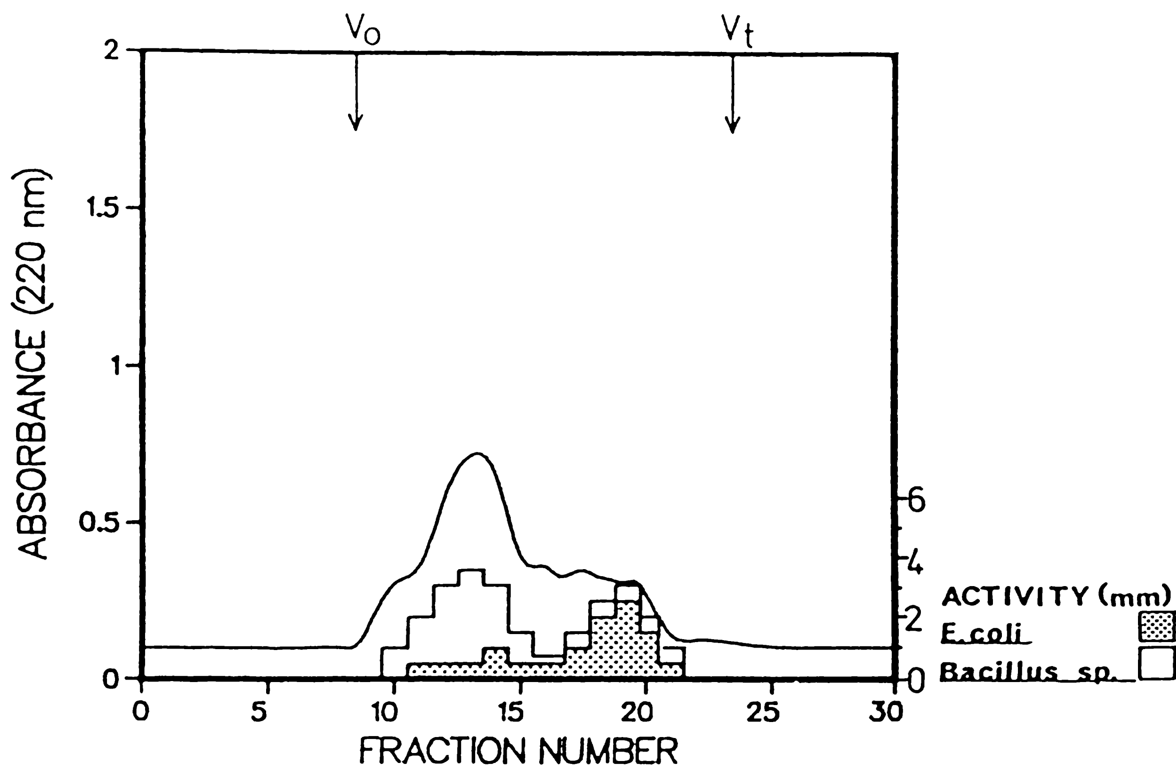


Fig. 4-4: Gel Filtration Chromatography of Fractions 131-170 from Fig. 4-1b
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 131-170 (Fig. 4-1b), desalted, freeze-dried and re-dissolved in 5 ml of 0.02 mol/l HCl, pH 1.7
Fraction Volume: 10 ml

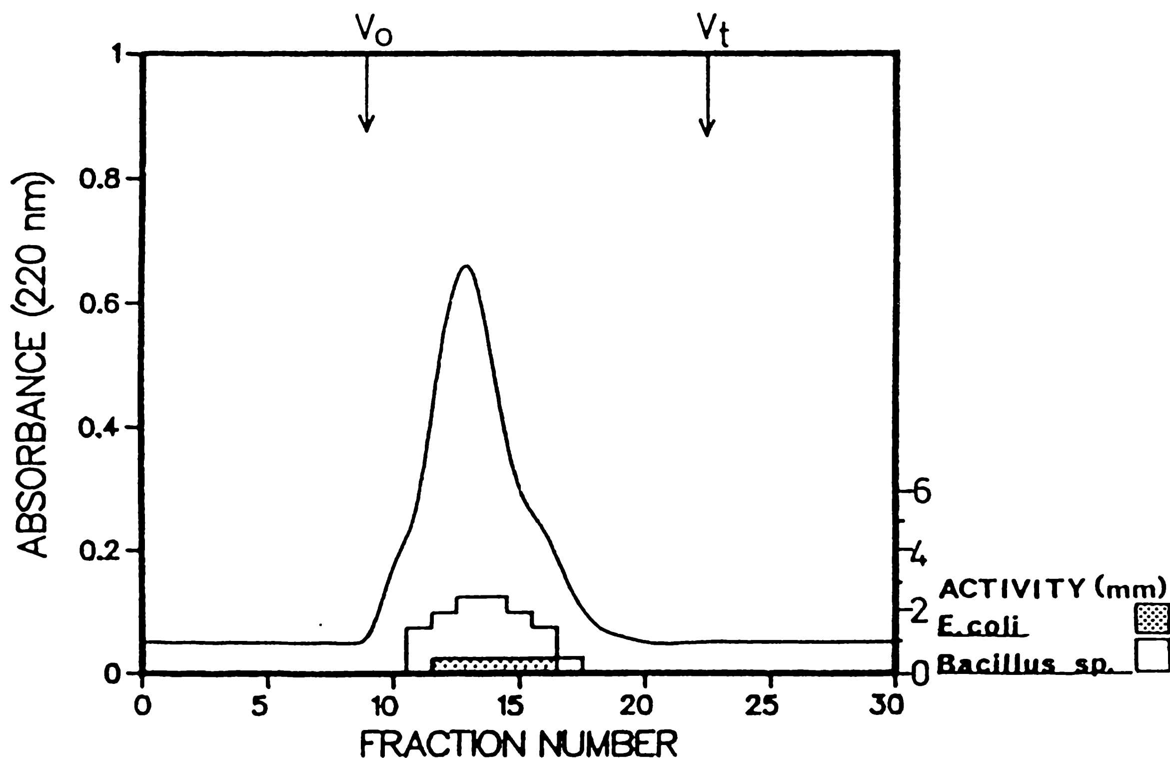


Fig. 4-5: Gel Filtration Chromatography of Fractions 10-16 from Fig. 4-4
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 10-16 (Fig. 4-4), evaporated to 5 ml
Fraction Volume: 10 ml

Fractions 10-16 (Fig. 4-4) were rotary evaporated to 5 ml and re-chromatographed through the column of Sephadex G 50 eluted with 0.02 mol/l HCl, pH 1.7. A broad asymmetrical peak with antibacterial activity resulted as shown in Fig. 4-5. Samples of 1 ml were taken from fractions 10, 12, 14, and 16, freeze-dried separately, and re-dissolved in SDS sample buffer. They were examined by SDS electrophoresis at a constant current of 40 mA for 3 hours. Several bands were visible in each of the fractions (see Plate 4-5), but there was a predominant band which migrated the same distance as a lysozyme-like protein extracted from sheep thymus (discussed in Chapter 6).

Fractions 17-21 (from Fig 4-4) were rotary evaporated to 5 ml and re-chromatographed through a column of Sephadex G 25 SF eluted with 0.02 mol/l HCl. A sharp symmetrical peak was eluted as shown in Fig. 4-6, in a position corresponding to the void volume of the column. A 1 ml sample was taken from fraction 14, freeze-dried, re-dissolved in SDS sample buffer and examined by SDS electrophoresis. The results are shown in Plate 4-5. Two major bands of low molecular weight were present. In comparing Plate 4-3 with Plate 4-5 the seminalplasmin fraction from the Sephadex G 25 (Fig. 4-6) has a band of low molecular weight present which was not seen earlier from the seminalplasmin fraction from the Sephadex G 75 (Fig. 4-2b). This band may represent a significant disaggregation from the higher molecular weight material. However a more likely explanation is that this band was not seen before as it was lost during the dialysis step. This band has an estimated molecular weight of 8 000 and the dialysis tubing used had a nominal retention of 10 000 daltons.

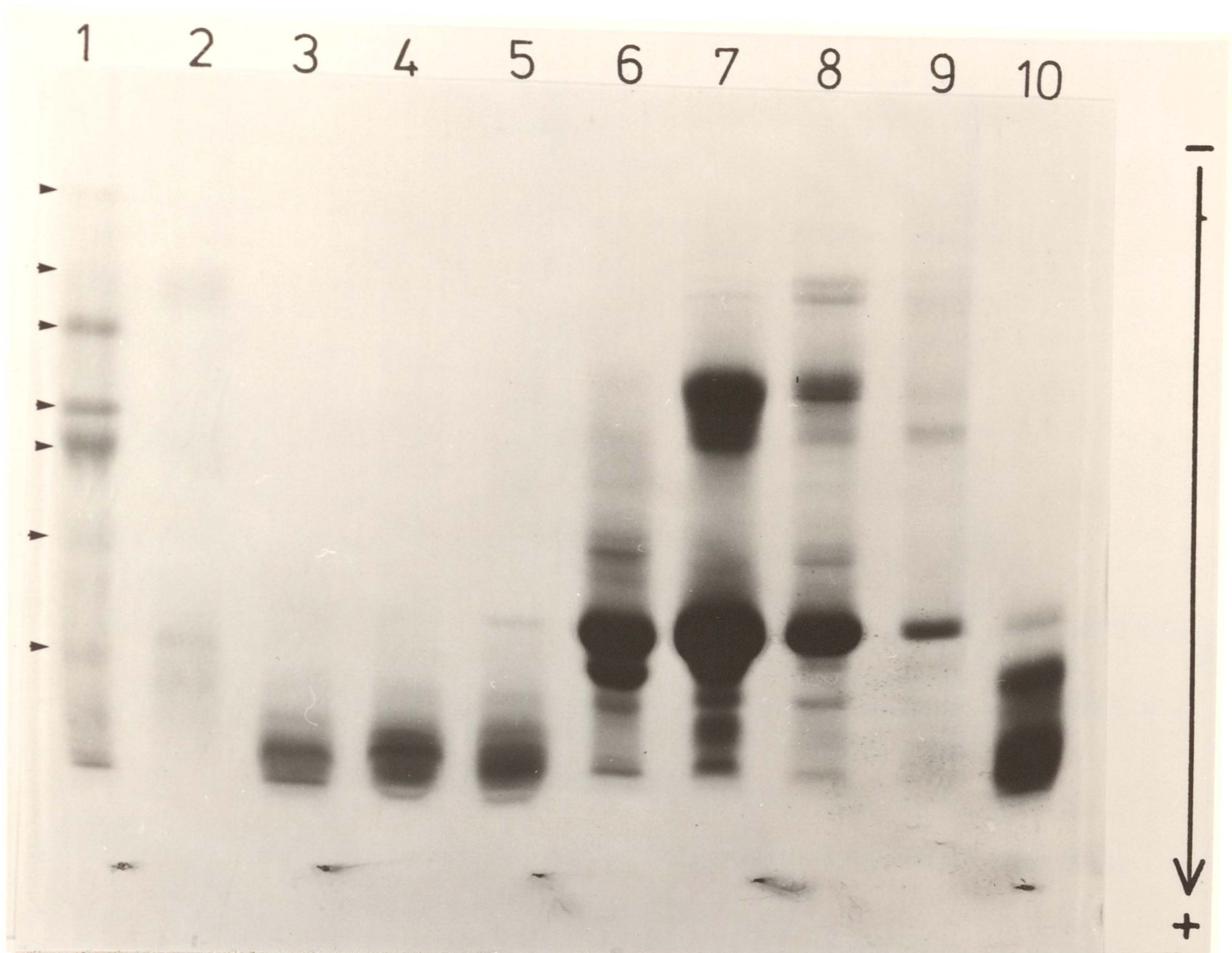


Plate 4-5: SDS Electrophoresis

1 - α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin standards.

2 - not relevant

3 - not relevant

4 - not relevant

5 - not relevant

6 - fraction 16 from Fig. 4-5

7 - fraction 14 from Fig. 4-5

8 - fraction 12 from Fig. 4-5

9 - fraction 10 from Fig. 4-5

10- fraction 14 from Fig. 4-6

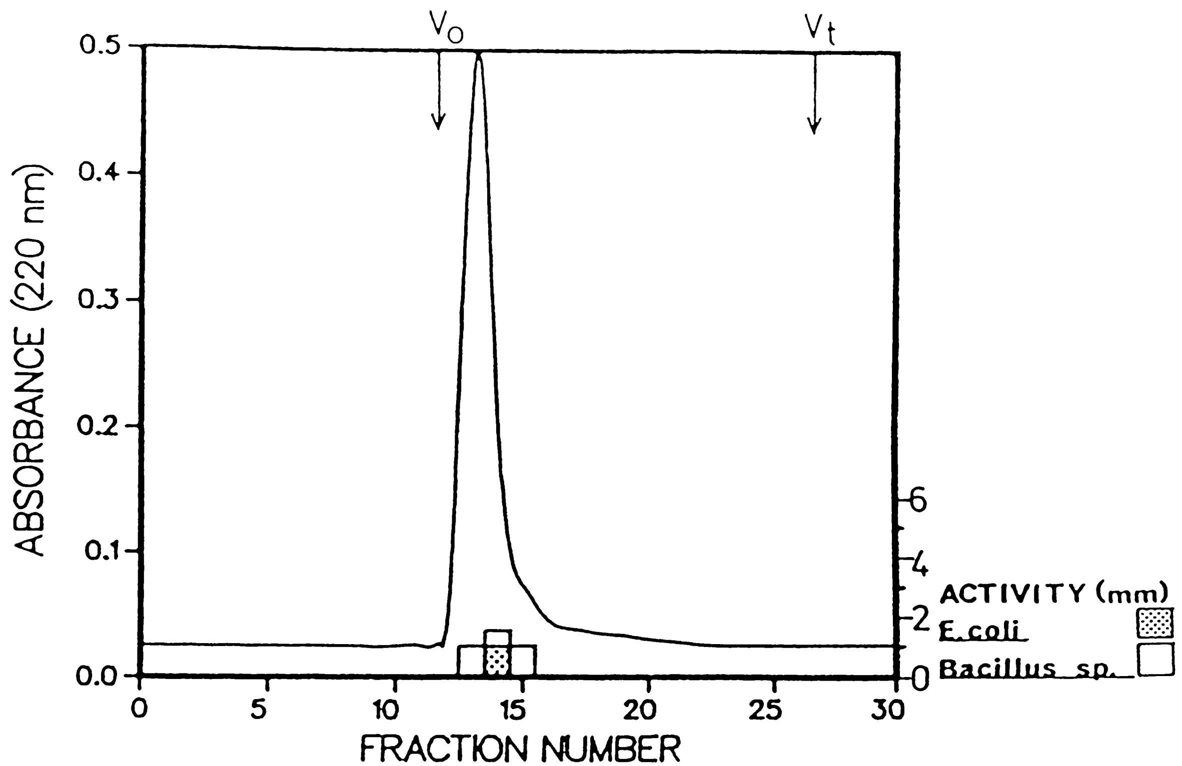


Fig. 4-6: Gel Filtration Chromatography of Fractions 17-21 from Fig. 4-4
Gel: Sephadex G 25 SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 17-21 (Fig. 4-4), evaporated to 5 ml
Fraction Volume: 10 ml

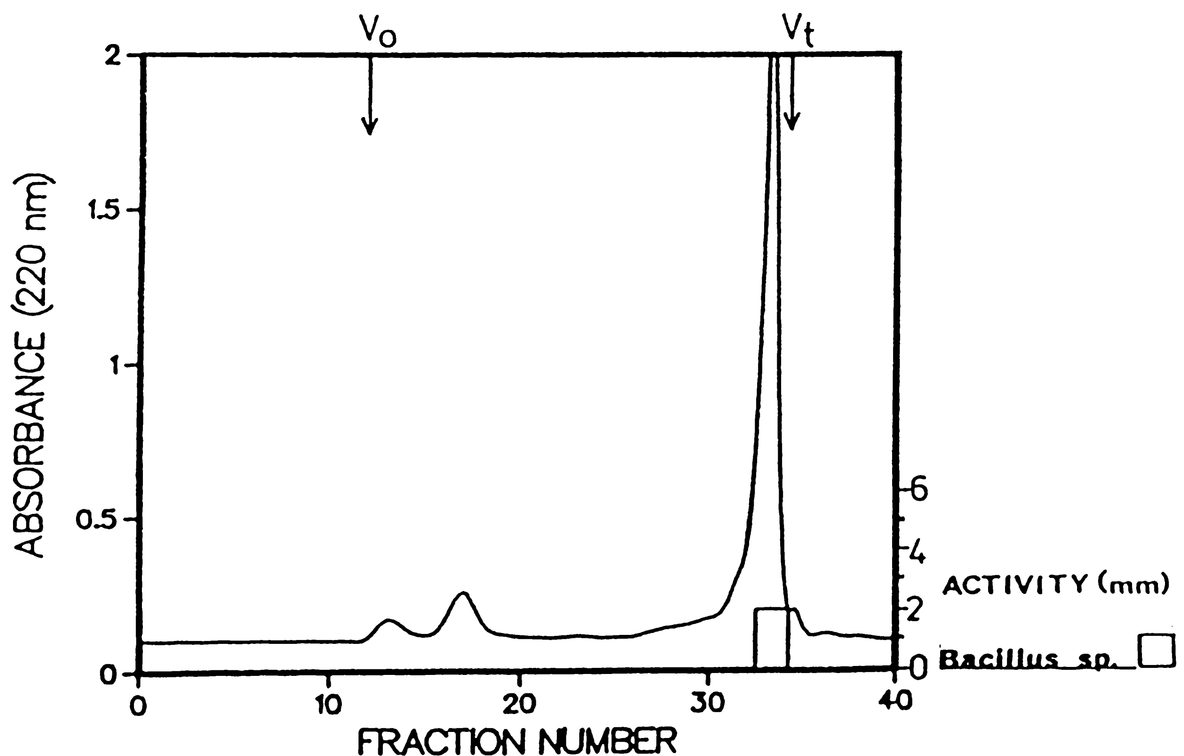


Fig. 4-7: Gel Filtration Chromatography (HPLC) of Fractions 13-15 from Fig. 4-6
Gel: TSK 3000SWG
Eluent: Distilled water
Sample: Fractions 13-15 (Fig. 4-6), evaporated to 2 ml and 1 ml of this applied to the column
Fraction Volume: 6 ml

As SDS electrophoresis showed the presence of more than one band in the peaks shown in Figs. 4-5 and 4-6, further gel filtration chromatography was carried out to obtain homogeneous samples. Fractions 13-15 (Fig. 4-6) were rotary evaporated to 2 ml, and 1 ml of this was chromatographed by HPLC on a column of TSK 3000SWG gel filtration medium eluted with water. A large active peak was eluted from the column near the bed volume (see Fig. 4-7). It was concluded that some material was retained on the column as the non-active peak was too small to account for the impurities (the additional higher molecular weight band seen in fraction 14, Plate 4-5) in the applied sample. A 1 ml sample from fraction 34 was freeze-dried, dissolved in SDS sample buffer and separated by SDS electrophoresis. The results are shown in Plate 4-6. A single band running near the solvent front was the only protein present. This band has an estimated molecular weight of 7 500, and was presumed to be homogeneous seminalplasmin (probably a more purified form than that obtained by Reddy and Bhargava (1979)). This band corresponded to the low molecular weight band present in Plate 4-5 from the column of Sephadex G 25 shown in Fig. 4-6. The band of higher molecular weight had probably been largely absorbed onto the gel filtration column. It was possible that this band was also antibacterial. However as it was present in the earlier purification procedure after dialysis (Plate 4-3) and did not exhibit any antibacterial activity, it was probably an inactive impurity.

The fractions of higher molecular weight (11-17) from Fig. 4-5 were rotary evaporated to 2 ml, and 1 ml of this chromatographed by HPLC on a column of TSK 3000SWG eluted with water. The results are shown in Fig. 4-8. Activity against the Bacillus sp. was detected in the peak at its bed volume (fractions 27-28). A 1 ml sample from fraction 28 was

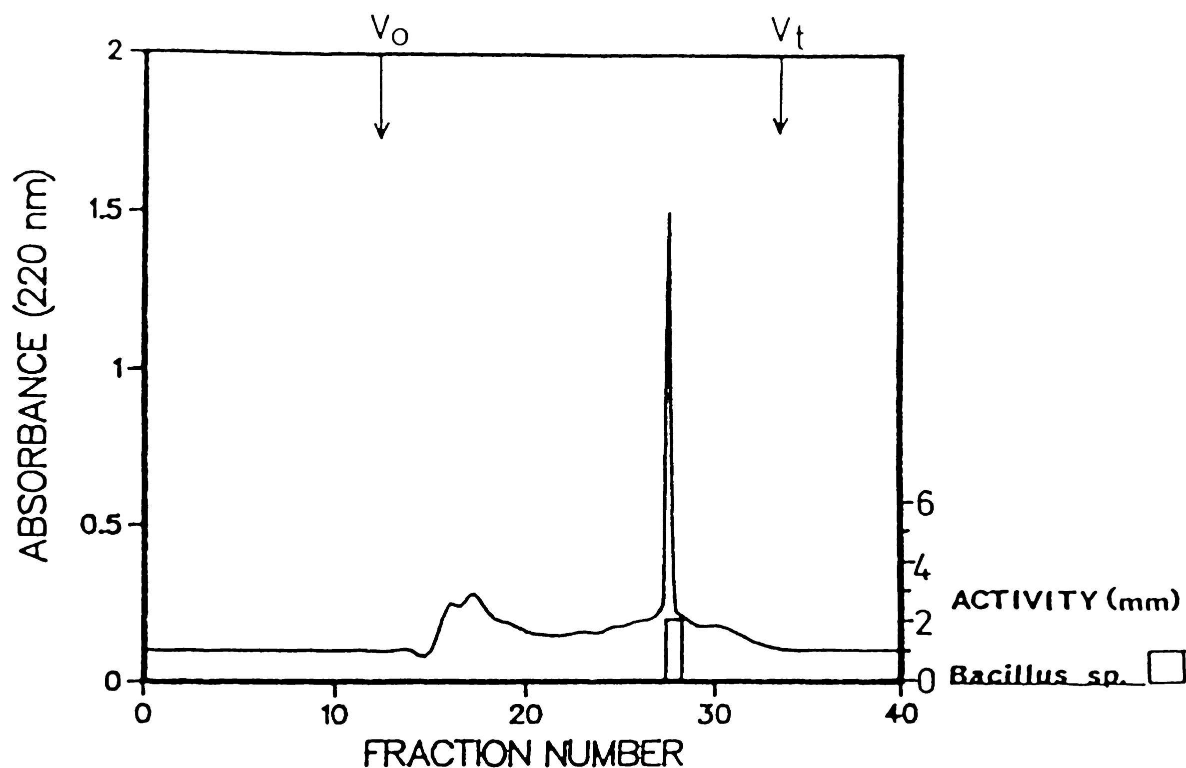


Fig. 4-8: Gel Filtration Chromatography (HPLC) of Fractions 11-17 from Fig. 4-5
Gel: TSK 3000SWG
Eluent: Distilled water
Sample: Fractions 11-17 (Fig. 4-5), evaporated to 2 ml and 1 ml of this applied to the column
Fraction Volume: 6 ml

freeze-dried, dissolved in SDS sample buffer and separated by SDS electrophoresis. The results are shown in Plate 4-6. The migration of the major band was similar to that of the bovine lysozyme isolated by Eschenbruch (1980).

4.6 Estimation of Molecular Weights of the Antibacterial Proteins in Seminal Plasma.

In the presence of SDS the electrophoretic mobility of proteins in polyacrylamide gels is determined by the molecular weight of their polypeptide chains and to their extent of being "coated" with a negative charge.

The discontinuous SDS electrophoresis method of Laemmli (1970) was used for molecular weight determination. The results are shown in Plate 4-6a and b. The migration distances (calculated as R_f values) of the standards were plotted against the logarithm of their molecular weights to obtain a standard curve (see Fig. 4-9). Using this method, the molecular weight of seminalplasmin was calculated to be 7 500, and that of the lytic protein from fraction 28 (Fig. 4-8) estimated to be 15 000. This latter value is very similar to that obtained by Eschenbruch (1980) for bovine lysozyme isolated from seminal plasma.

The molecular weight of seminalplasmin is lower than that reported by Reddy and Bhargava (1979) (10 600) using the same electrophoretic method.

Plate 4-6a: SDS Electrophoresis

1 - α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin standards

2 - fraction 28 from Fig. 4-8

3 - fraction 33 from Fig. 4-7

4 - fraction 34 from Fig. 4-7

5 - bovine lysozyme from sheep thymus

6 - bovine lysozyme from sheep thymus

7 - fraction 34 from Fig. 4-7

8 - not relevant

9 - not relevant

10- α-lactalbumin standard

Plate 4-6b: SDS Electrophoresis

1 - α-lactalbumin standards

2 - not relevant

3 - not relevant

4 - fraction 34 from Fig. 4-7

5 - bovine lysozyme from sheep thymus

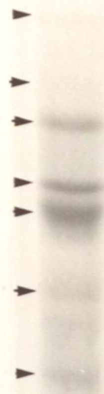
6 - bovine lysozyme from sheep thymus

7 - fraction 34 from Fig. 4-7

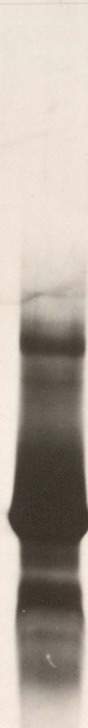
8 - fraction 33 from Fig. 4-7

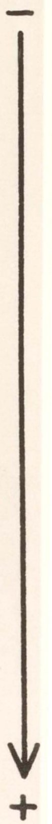
9 - fraction 28 from Fig. 4-8

10- α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin standards

1
2
3
4
5
6
7
8
9
10
–
+


1
2
3
4
5
6
7
8
9
10
–
+





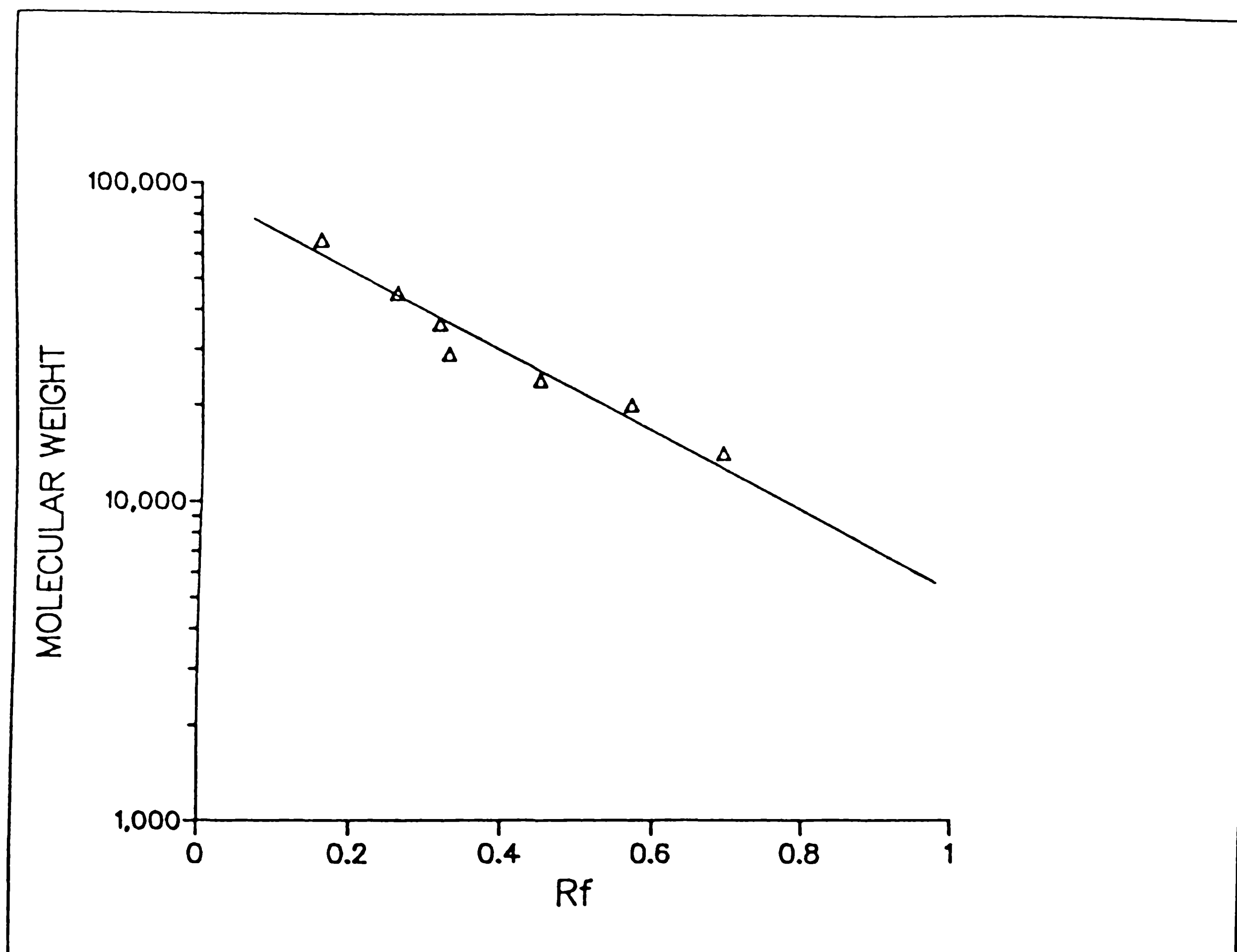


Fig. 4-9: Calibration Curve of Standards Run on SDS Electrophoresis

4.7 Determination of Lysozyme Activity

Both seminalplasmin and the lysozyme-like protein were assayed for lytic activity. The measurement of lytic activity was based on the lyso-plate method of Osserman and Lawler (1966) where the lysozyme-like activity of a sample is assayed by the lysis of pre-grown *Micrococcus lysodeikticus* cells in nutrient agar plates. The lyso-plate method of Gosnell *et al*. (1975), based on the lysis of lyophilised *Micrococcus lysodeikticus* cells, was also used.

Samples of 50 µl were taken of seminalplasmin (fraction 34, Fig. 4-7) and the lysozyme-like protein (fraction 28, Fig. 4-8). These were placed in test wells of various plates as outlined below. The plates were incubated at 37°C for 12 hours and the zones of clearing were measured. A standard of egg white lysozyme (0.5 mg/ml) was also tested.

	Activity (mm)		
	seminalplasmin	lysozyme-like protein	egg white lysozyme
Pre-grown M. lysodeikticus plates	0	2	4
Lyophilised M. lysodeikticus plates	0	3	7
E. coli seeded plates	2	0	0.5
Bacillus sp. seeded plates	3	3	5

Seminalplasmin showed no lytic activity whereas the lysozyme-like protein gave definite clearings in the lyso-plate assays.

4.8 Determination of Isoelectric Point

Isoelectric focusing was carried out using Servalyt-Precotes with a focusing range of pH 3-10. Samples of 1 ml from fraction 34 containing seminalplasmin (Fig. 4-7) and fraction 28 containing the lysozyme-like protein (Fig. 4-8) were reduced by rotary evaporation to 50 µl. Samples of 10 µl were applied to the gel which was run at a constant power of 4 watts for 2 hours at which point the standards and ampholine bands were clearly visible. The results are shown in Fig. 4-10.

The lysozyme-like protein gave a clear band at the cathode indicating an isoelectric point greater than pH 10. This was not unexpected as egg white lysozyme has an isoelectric point between pH 10.5-11.0 (Salton, 1957). The seminalplasmin fraction gave no detectable band even after repeated attempts with high sample loadings.

4.9 Conclusions

The antibacterial activity of seminalplasmin was not attributable to the presence of the polyamines spermine and spermidine. However seminalplasmin isolated by Reddy and Bhargava (1979) was almost certainly a heterogeneous product. Although Reddy and Bhargava used an additional step, an affinity gel to remove ribonuclease, most of the other compounds found in the present study were of the wrong molecular weight to be ribonuclease. The differences they obtained in the molecular weight values for seminalplasmin using various methods suggest heterogeneity. This is further supported by the results obtained during this study. The final purification step using HPLC resulted in a single band on SDS electrophoresis with an approximate molecular weight of 7 500 which is lower than that published (10 600) using the same electrophoretic method.

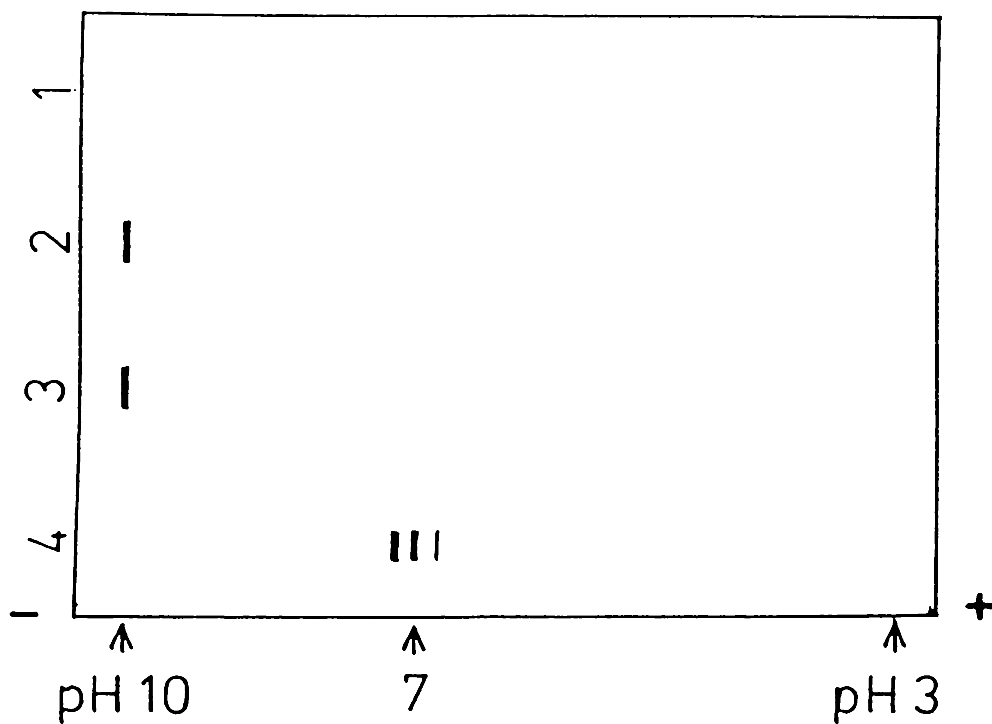


Fig. 4-10: Isoelectric Focusing
1 - fraction 34 from Fig. 4-7
2 - fraction 28 from Fig. 4-8
3 - lysozyme standard
4 - myoglobin standard

The isoelectric point of the purified seminalplasmin could not be determined. This may be due to its highly cationic nature and small size. In isoelectric focusing, peptides below a molecular weight of 10 000 are increasingly difficult to fix and stain, and are often washed out of the gel (Righetti and Chillemi, 1978). Reddy and Bhargava obtained a value of 9.8 for the isoelectric of point seminalplasmin. This was probably due to the heterogeneity of their sample, which as a result of its increase in size, allowed it to be fixed and stained after isoelectric focusing. The presence of a lysozyme-like protein was not mentioned by Reddy and Bhargava. However lysozyme is not very active against E. coli and this may explain why it was not detected. This lysozyme-like protein is removed from the column of CM Sephadex C 50 with seminalplasmin, and is also eluted close to seminalplasmin from the Sephadex G 75 gel filtration column. It is likely that the seminalplasmin isolated by Reddy and Bhargava would have contained some lysozyme. Using their method of purification, bands corresponding to the lysozyme-like protein were detected on SDS electrophoresis (Fraction C, Plate 4-3). The presence of lysozyme may be important in the in vivo activity of seminalplasmin, it possibly acting synergistically in the non-specific immune response.

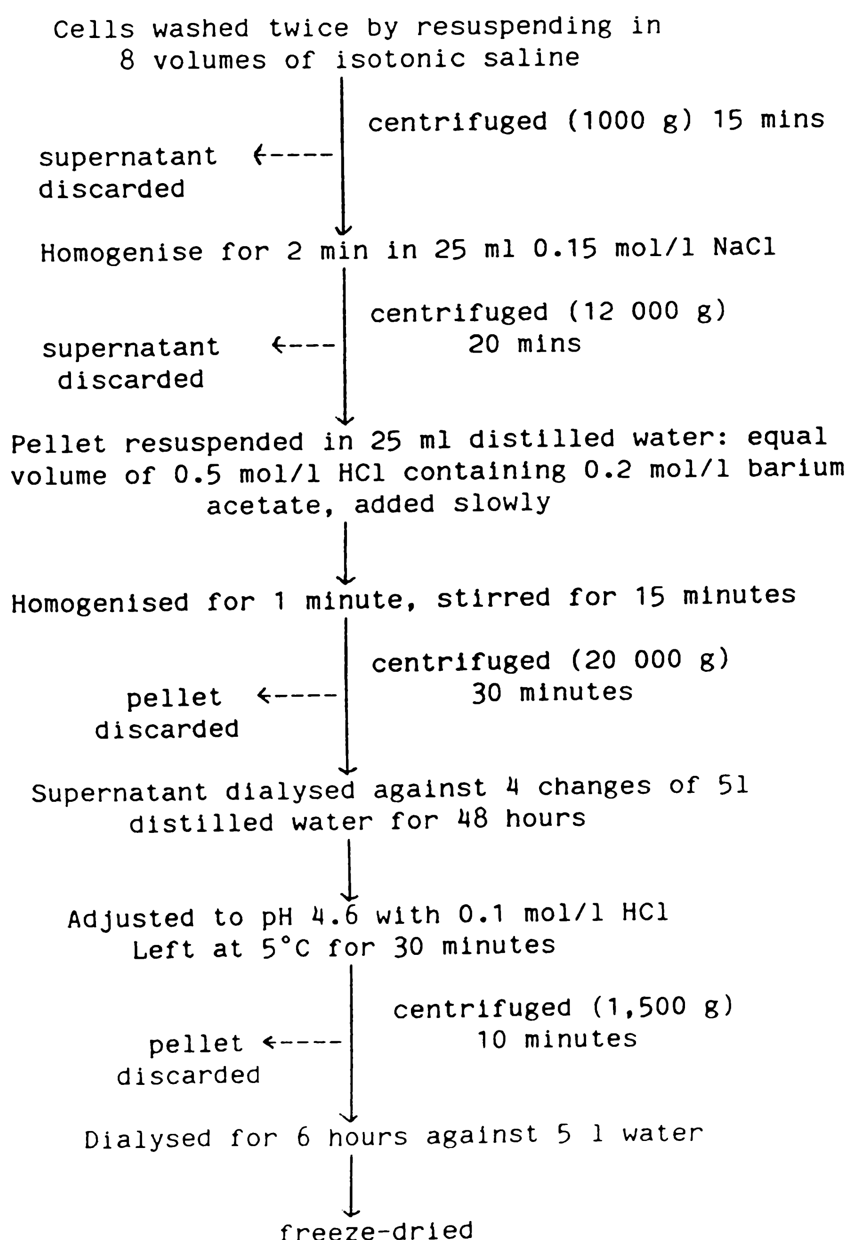
CHAPTER FIVE

THE CHARACTERISTION OF THE ANTIBACTERIAL COMPOUNDS FROM THE CELLS IN MILK

5.1 Introduction

In 1971, Hibbitt and coworkers reported the isolation of antibacterial cationic proteins from the cells in bulk milk samples. The cell fraction consisted of a large proportion of epithelial cells as well as a variety of other cells which included neutrophils, basophils, lymphocytes and monocytes.

Cationic proteins were extracted from the milk cells by the following method:



Any antibiotics which may have been present in the milk would have been removed from the cationic proteins during the process of extraction and purification.

5.2 Some Properties of the Isolated Antibacterial Cationic Proteins

Hibbitt and coworkers described several characteristics of the cationic proteins isolated from bovine milk cells. Polyacrylamide gel electrophoresis showed the presence of at least 9 components with isoelectric points between 7 and 9, and one component with an isoelectric point above 9.

The cationic protein fraction showed antibacterial activity against strains of Staphylococcus aureus, with concentrations of 1.0 µg/ml producing 50% inhibition of growth. Failure to lyse Micrococcus lysodeikticus in assays involving a 90 minute incubation suggested that lysozyme was absent from this extract. This was also consistent with electrophoresis results. As lysozyme has an isoelectric point of 10.5 to 11.0 it would have appeared as a band migrating towards the cathode at pH 9.

No loss of antibacterial activity was observed after heating the extract to 70°C for 30 minutes, whereas at higher temperatures the activity diminished. Only 10% of original activity was retained after incubation at 100°C for 10 minutes and it was almost completely destroyed after 30 minutes.

No further attempts were made to establish the active component or components. The initial aim in the current investigation was to determine whether spermine and/or spermidine was responsible for the antibacterial activity, and if not, then to isolate the active

components present.

5.3 Extraction and Identification of the Cationic Proteins from Milk Cells

The milk cells were obtained from a separator at a local dairy factory and the cationic proteins were extracted as outlined above (Section 5.1). The final product exhibited antibacterial activity against *E. coli* and the *Bacillus* species which were used as test organisms during this study. The extract displayed no lytic activity against *Micrococcus lysodeikticus* using the lyso-plate assay method.

After electrophoresis at pH 3.0 there were at least nine bands which migrated to the cathode, whereas at pH 7.0 definite bands were hard to distinguish but were still travelling slowly towards the cathode. At pH 9.0 there did not appear to be any protein moving toward the cathode, however some protein was stationary suggesting an isoelectric point near pH 9. These electrophoresis results were very similar to those of Hibbitt and coworkers.

The milk cell cationic proteins were found to be extremely heat-stable. There was no change in antibacterial activity after heating to 70°C for 30 minutes and only a slight loss of activity after heating to 100°C for 10 minutes. A significant amount of precipitation occurred after heating to 100°C but this did not seem to affect the antibacterial activity. These results are different from those of Hibbitt and coworkers who reported a loss of 90% of original activity after heating at 100°C for 10 minutes. The reason for the difference in results is not clear. However, it is possible that variations in extraction procedure may have resulted in extracts of substantially different composition. In addition, the use by Hibbitt *et al*. (1971)

of Staphlyococcus aureus 305 and Streptococcus agalactiae S13 as test organisms may be responsible for differences in results. It is likely that different organisms exhibit dissimilar levels of sensitivity to the various inhibitory compounds.

5.4 Investigation of the Presence of Spermine in the Milk Cell Extract

Briggs (1982) found that spermine and spermine-peptide complexes were eluted near the bed volume of a Sephadex G 25 column.

Therefore a 5 ml sample of the milk cell extract in Tris buffer, 0.1 mol/l pH 7.2, was chromatographed through a column of Sephadex G 25 eluted with the same buffer, to see if any spermine could be separated from the extract. A non-dissociating buffer was used in order to distinguish between the presence of free spermine and protein-complexed spermine. Fig. 1 shows the elution and activity profiles. No active fractions were detected in the region of low molecular weight (fractions 20-30) where spermine would be expected to be eluted.

The experiment was repeated using a more dissociating buffer. A 5 ml sample of the milk cell extract in 0.02 mol/l HCl pH 1.7, was chromatographed through a column of Sephadex G 25 and eluted with 0.02 mol/l HCl. The results are shown in Fig. 2. Before assaying for antibacterial activity, 100 µl samples from each 10 ml fraction were neutralised by addition of 100 µl of 0.1 mol/l phosphate buffer pH 7. The elution profile and activity profile were identical to that obtained with Tris buffer, with no spermine being eluted in the region of low molecular weight.

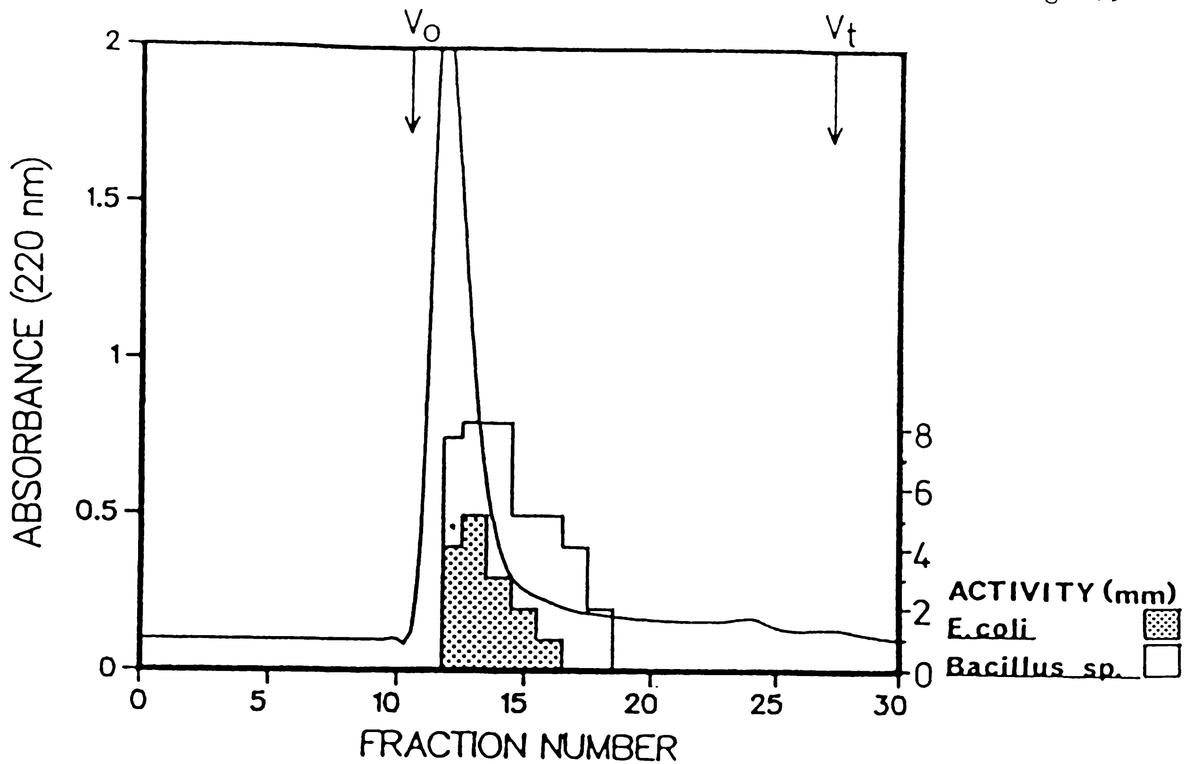


Fig. 5-1: Gel Filtration Chromatogrphy of the Milk Cell Extract
Gel: Sephadex G 25 SF
Eluent: 0.1 mol/l Tris buffer, pH 7.2
Sample: Milk cell extract dissolved in 5 ml of 0.1 mol/l Tris buffer, pH 7.2
Fraction Volume: 10 ml

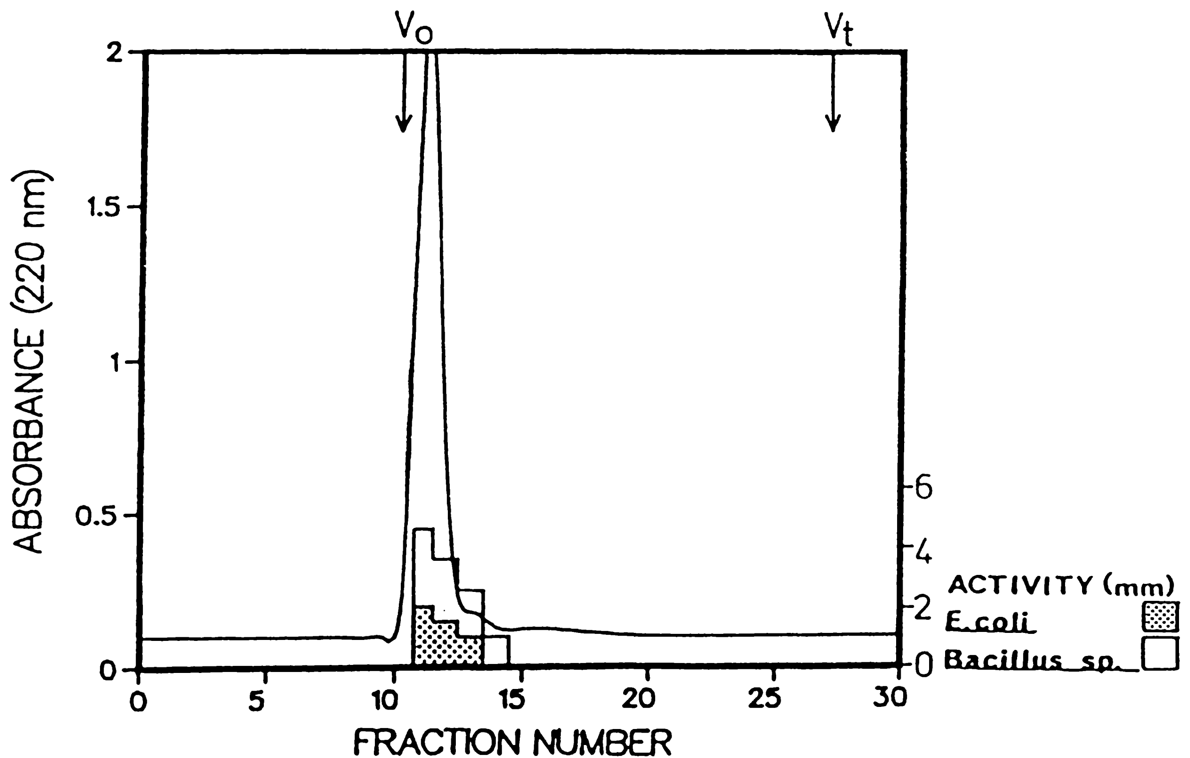


Fig. 5-2: Gel Filtration Chromatography of the Milk Cell Extract
Gel: Sephadex G 25 SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Milk cell extract dissolved in 5 ml of 0.02 mol/l HCl, pH 1.7
Fraction Volume: 10 ml

Ion exchange chromatography was also used in an attempt to isolate any spermine present in the milk cell extract. Briggs (1982) found that spermine was eluted from CM Sephadex C 25 ion exchanger in 0.1 mol/l phosphate buffer, pH 7 at a salt concentration of 0.78 mol/l NaCl. A 10 ml sample of milk cell extract dissolved in 0.1 mol/l phosphate buffer pH 7, was applied to a column of CM Sephadex C 25 ion exchanger which had been equilibrated with the same buffer. The column was washed with phosphate buffer until the absorbance at 220 nm of the effluent had returned to the baseline, and then eluted with 0.4 mol/l NaCl in 0.1 mol/l phosphate buffer pH 7. This was followed by a linear salt gradient from 0.4 to 2.0 mol/l NaCl in phosphate buffer pH 7, and a final wash of 0.1 mol/l NaOH to remove any remaining material. The results are shown in Fig. 5-3. It is known that the antibacterial activity of spermine is relatively unaffected by NaCl concentrations up to 0.4 mol/l, but above this it is partially lost and at 0.7 mol/l it is completely inhibited (Briggs, 1982). Fractions containing high salt concentrations were tested after dilution with 0.1 mol/l phosphate buffer pH 7, to eliminate the inhibiting effects of salt. The ion exchange chromatography resolved the milk cell extract into 3 active peaks. Fractions 4-7 were not adsorbed onto the gel and were eluted with the phosphate buffer. Fractions 18-34 were eluted by 0.4 mol/l NaCl and fractions 38-48 were eluted by the salt gradient at about 1.0 mol/l NaCl. The antibacterial activity in fractions 38-48 did not appear to be inhibited by the salt to any large extent.

To examine whether spermine was present in these fractions they were each re-chromatographed. Fractions 4-7 were evaporated to 10 ml and re-chromatographed on a column of Sephadex G 25 eluted with 0.02 mol/l HCl, pH 1.7 (Fig. 5-4). The salt present in fractions 18-34

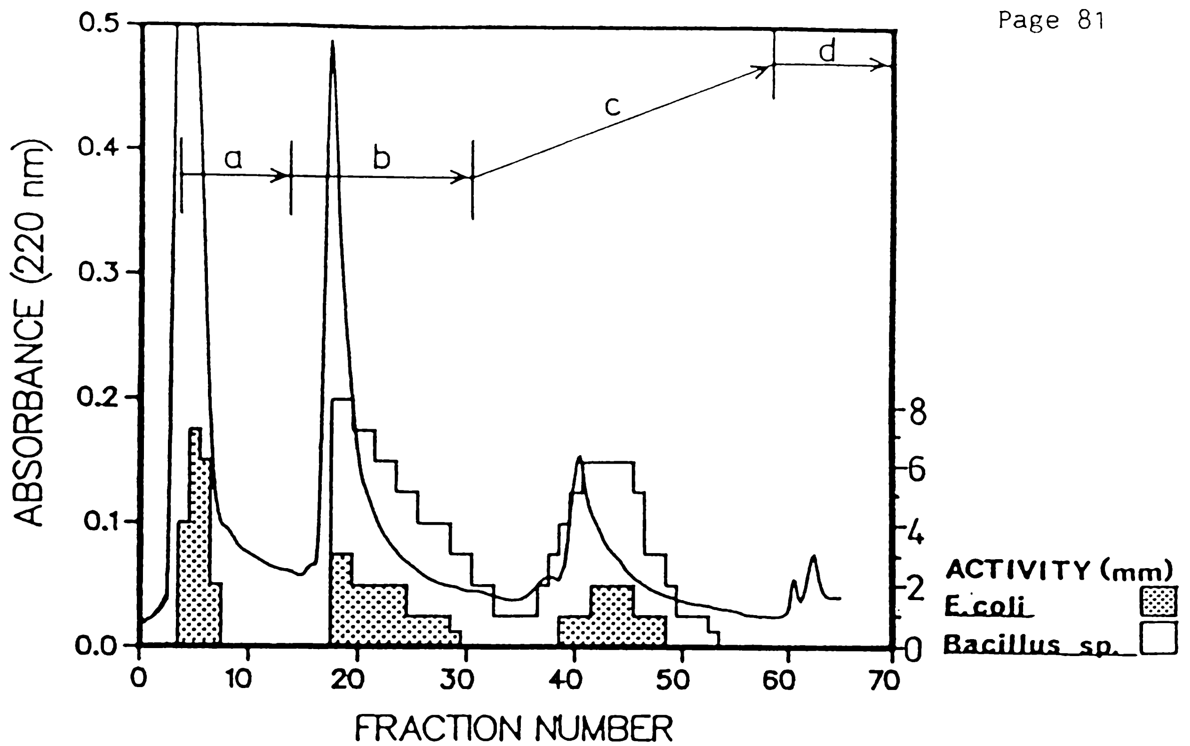


Fig. 5-3: Ion Exchange Chromatography of the Milk Cell Extract
Gel: CM Sephadex C 25
Buffer: 0.1 mol/l phosphate, pH 7
Sample: Milk cell extract dissolved in 10 ml of 0.1 mol/l phosphate buffer, pH 7
Elution Buffers: a- 0.1 mol/l phosphate buffer, pH 7; b- 0.4 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 7; c- salt gradient from 0.4 to 2 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 7; d- 0.1 mol/l NaOH
Fraction Volume: 10 ml

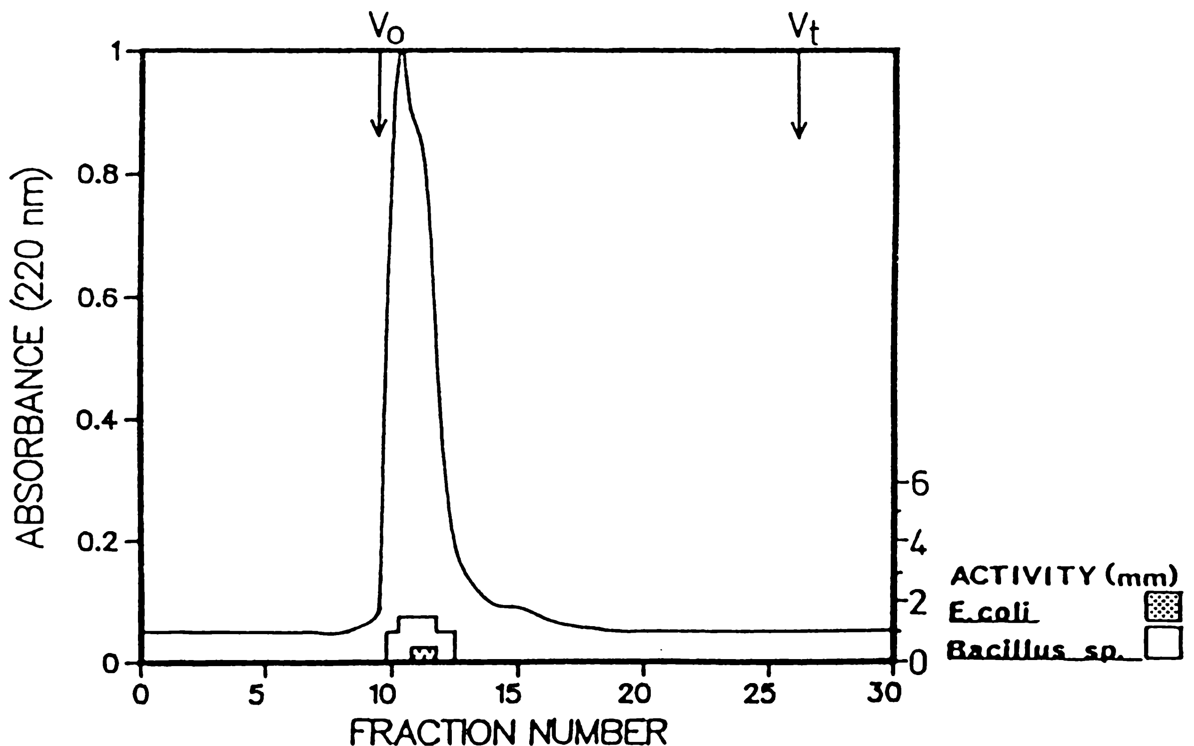


Fig. 5-4: Gel Filtration Chromatography of Fractions 4-7 from Fig. 5-3
Gel: Sephadex G 25 SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 4-7 (Fig. 5-3), evaporated to 10 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

and 38-48 had to be removed before they could be concentrated. Each pool was diluted 1:1 with distilled water and re-run onto the column of CM Sephadex C 25 ion exchange, washed with 0.1 mol/l phosphate buffer pH 7, and removed by 0.1 mol/l NaOH. The eluted fractions were immediately neutralised. Fractions 18-34 and 38-48 were separately evaporated down to 10 ml, adjusted to pH 1.7 and chromatographed on a column of Sephadex G 25 eluted with 0.02 mol/l HCl pH 1.7. The results are shown in Figs. 5-5 and 5-6. The elution profiles of Figs. 5-4, 5-5 and 5-6 were very similar, having a large void volume peak which was active. There was no indication of spermine being present in the fractions of low molecular weight.

It was concluded that spermine was not present in the milk cell extract in a free state. To find if spermine was present in a bound state samples were hydrolysed and run on thin-layer chromatography. The active fractions from the gel filtration chromatographs of fractions 4-7, 18-34 and 38-48 were respectively pooled and freeze-dried. Samples (2 mg), from each of the fractions were hydrolysed for 24 hours by refluxing in concentrated HCl. The hydrolysed samples were evaporated to dryness. These were then dissolved in 5 ml of distilled water and evaporated to dryness. This was repeated 3 times to remove the HCl. Finally the hydrolysates were dissolved in distilled water (100 µl) and samples taken for thin-layer chromatography. A 2 mg sample of lysozyme with 1 mg of spermine was also hydrolysed and run as a standard to examine whether the spermine is still detectable and not bound by the hydrolysed product of this cationic protein. Fig. 5-7 shows the ninhydrin-stained gel. There was no evidence of the presence of spermine or spermidine in the hydrolysates of fractions 4-7, 28-34 and 38-48, even when grossly overloaded. However spermine was easily

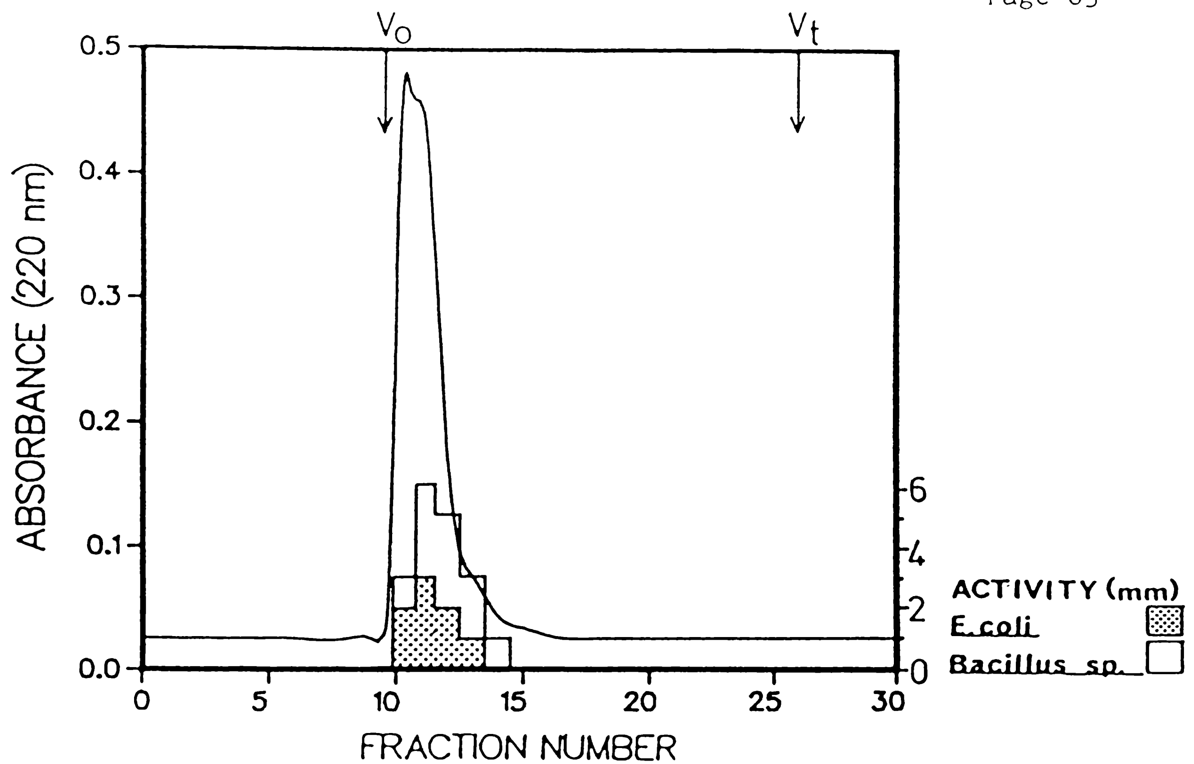


Fig. 5-5: Gel Filtration Chromatography of Fractions 18-34 from Fig. 5-3
Gel: Sephadex G 25 SF
Eluent: 0.02 mol/l HCl
Sample: Fractions 18-34 (Fig. 5-3), desalted, evaporated to 10 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

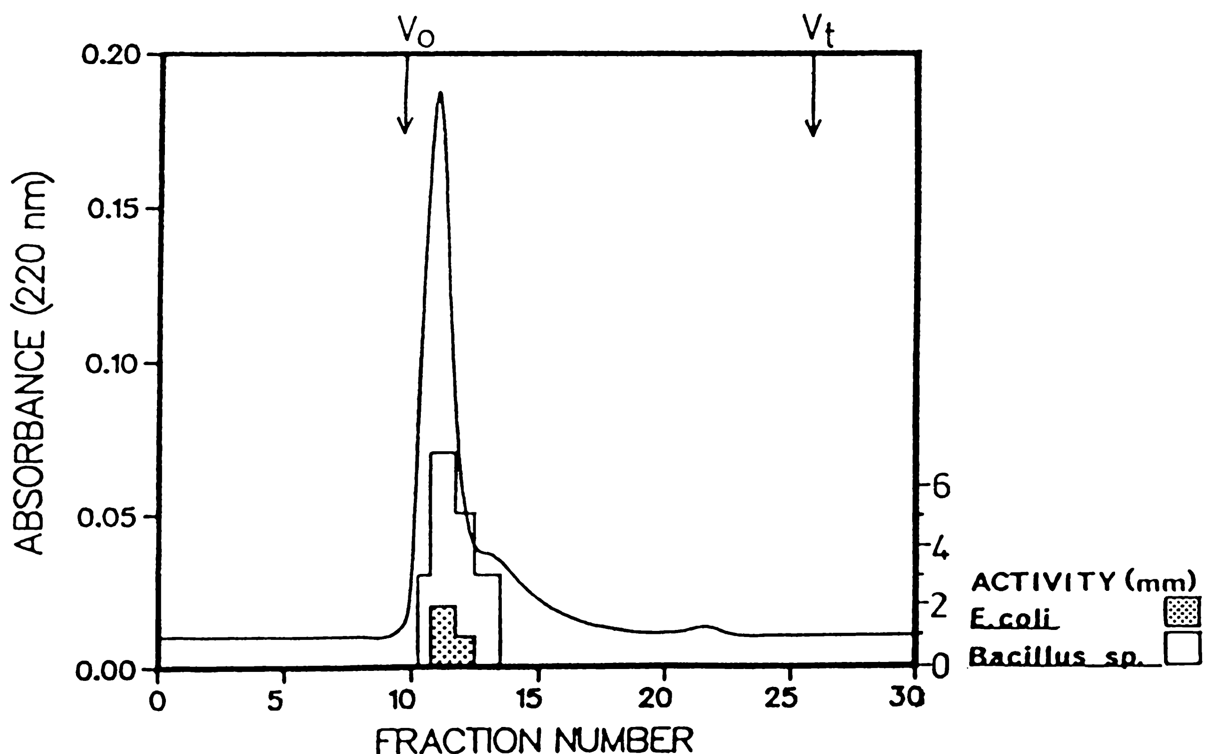


Fig. 5-6: Gel Filtration Chromatography of Fractions 38-48 from Fig. 5-3
Gel: Sephadex G 25 SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 38-48 (Fig. 5-3), desalted, evaporated to 10 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

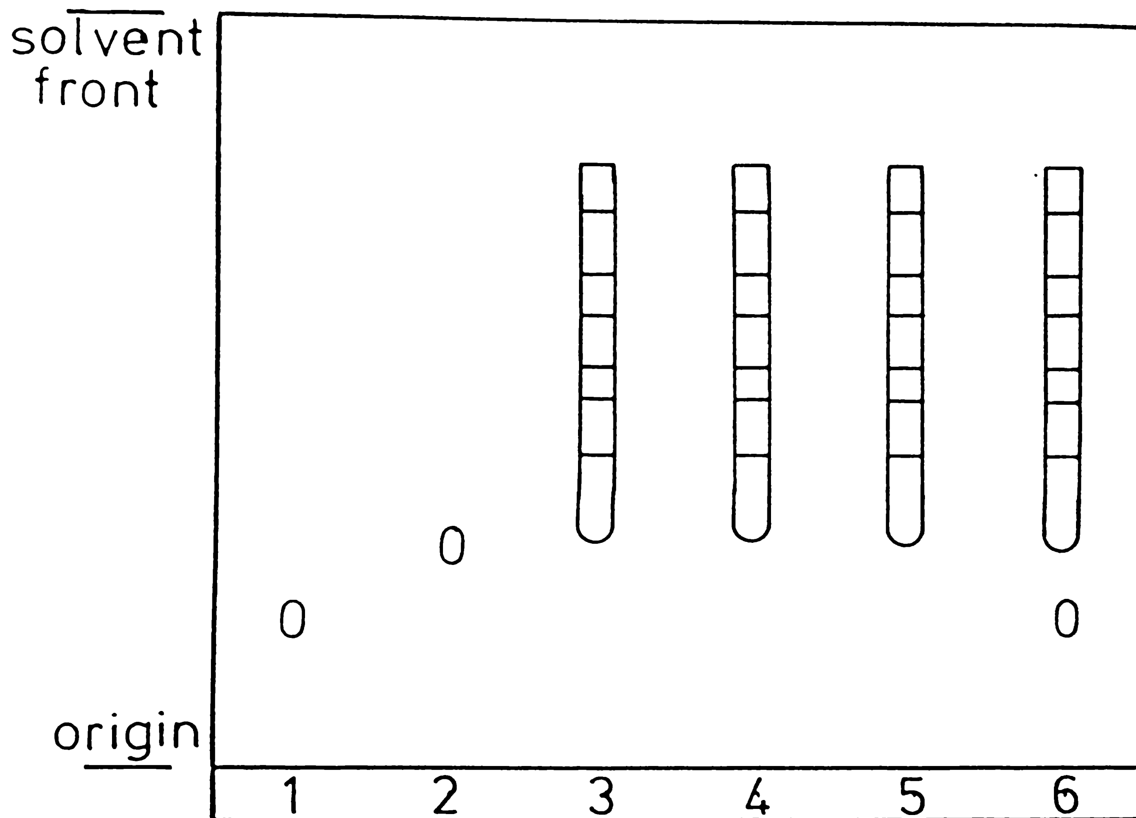


Fig. 5-7: Thin-layer Chromatography
1 - spermine standard
2 - spermidine standard
3 - fractions 10-15 from Fig. 5-4
4 - fractions 10-15 from Fig. 5-5
5 - fractions 10-15 from Fig. 5-6
6 - hydrolysed lysozyme and spermine standard

detectable in the hydrolysed lysozyme-spermine standard. It is possible that spermine was present but below the level of detection when stained on thin-layer chromatography. In such a case however, spermine would not be expected to have a significant contribution to the overall activity. Spermine at a concentration of 0.5 mg/ml is detectable on thin-layer chromatography when applied as a 2 µl sample. The maximum level that could be present in the hydrolysed fraction without being detected would be less than 1.25%, which is calculated using 0.5 mg/ml as the lowest level of detection.

Thus it was concluded that spermine is not present in the milk cell extract. Therefore investigations were directed towards isolating the antibacterial components present.

5.5 Isolation of the Active Components from the Milk Cell Extract

Samples of the active fractions 4-7, 18-34 and 38-48 from the ion exchange chromatography of the milk cell extract (Figs. 5-4, 5-5 and 5-6) were separated by SDS electrophoresis (see Plate 5-1) and cationic discontinuous electrophoresis (see Plate 5-2), to examine the degree of purification. Both electrophoresis gels showed that the fractions from the ion exchange chromatography were multibanded with many common bands, indicating that little purification was achieved by this method. The SDS gel also showed that there is a large amount of protein with molecular weights much larger than the lysozyme standard.

Gel filtration chromatography with Sephadex G 75 was used in an attempt to remove the higher molecular weight proteins. The remaining freeze-dried material of fractions 4-7, 18-34 and 38-48 were separately dissolved in 5 ml of 0.02 mol/l HCl pH 1.7 and chromatographed on a

Plate 5-1: SDS Electrophoresis

1 - lysozyme standard

2 - milk cell extract

3 - fractions 10-15 from Fig. 5-4

4 - fractions 10-15 from Fig. 5-5

5 - fractions 10-15 from Fig. 5-6

6 - fractions 10-15 from Fig. 5-6

Plate 5-2: Cationic Electrophoresis

1 - fractions 10-15 from Fig. 5-6

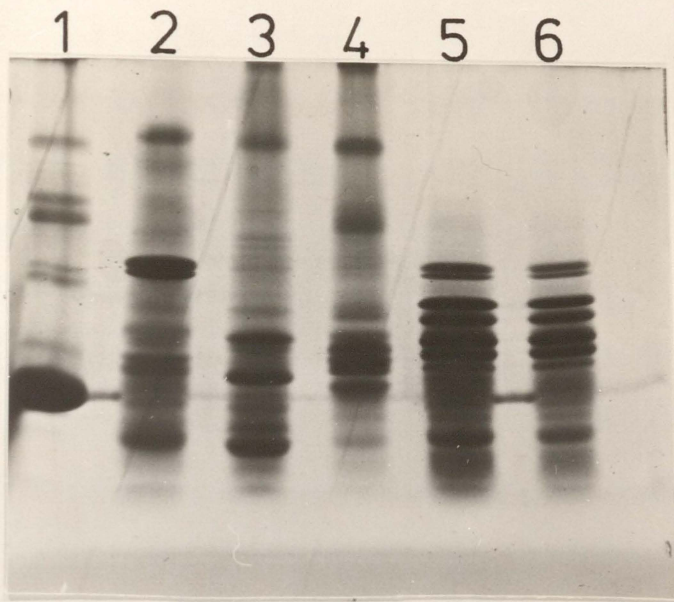
2 - fractions 10-15 from Fig. 5-5

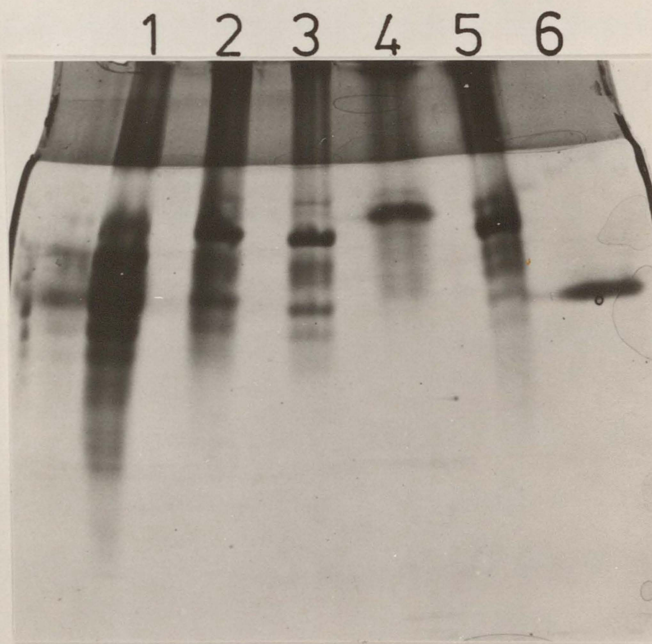
3 - fractions 10-15 from Fig. 5-5

4 - fractions 10-15 from Fig. 5-4

5 - milk cell extract

6 - lysozyme standard


1 2 3 4 5 6
−
+


1 2 3 4 5 6
+
−

column of Sephadex G 75 eluted with 0.02 mol/l HCl. The results are shown in Figs. 5-8a, b and c. The elution profiles were all similar in that there was a void volume peak followed by a broad peak which ran to the bed volume. Activity was generally associated with the broad peak, but activity was also found in the void volume peak in Fig. 5-8b. The active fractions were pooled, {fractions 15-30 (Fig. 5-8a), fractions 12-14 and 15-30 (Fig. 5-8b) and fractions 16-27 (Fig. 5-8c)} and freeze-dried. Samples from these freeze-dried fractions were separated by SDS electrophoresis. The stained gels revealed numerous bands, in a similar pattern to that previously found. This was not unexpected as the very broad peaks obtained from the chromatography with the Sephadex G 75 suggested that many proteins were eluting together.

Conclusions

The results demonstrate that the milk cell extract is a complex mixture of proteins, which remain largely aggregated during conventional gel filtration and ion exchange chromatography. In an attempt to dissociate these aggregates, a variety of protein denaturing reagents were included in the chromatography eluents.

5.6 Gel Filtration Chromatography in the Presence of SDS

Exposure to sodium dodecylsulphate (SDS) causes denaturation of proteins and dissociation of protein-protein interactions, by reducing inter- and intramolecular hydrophobic interactions (Weber and Osborn, 1969; Fish *et al*., 1970). The addition of sulphydryl reagents (e.g.:2-mercaptoethanol) can cause further molecular disruptions by cleaving disulphide bonds.

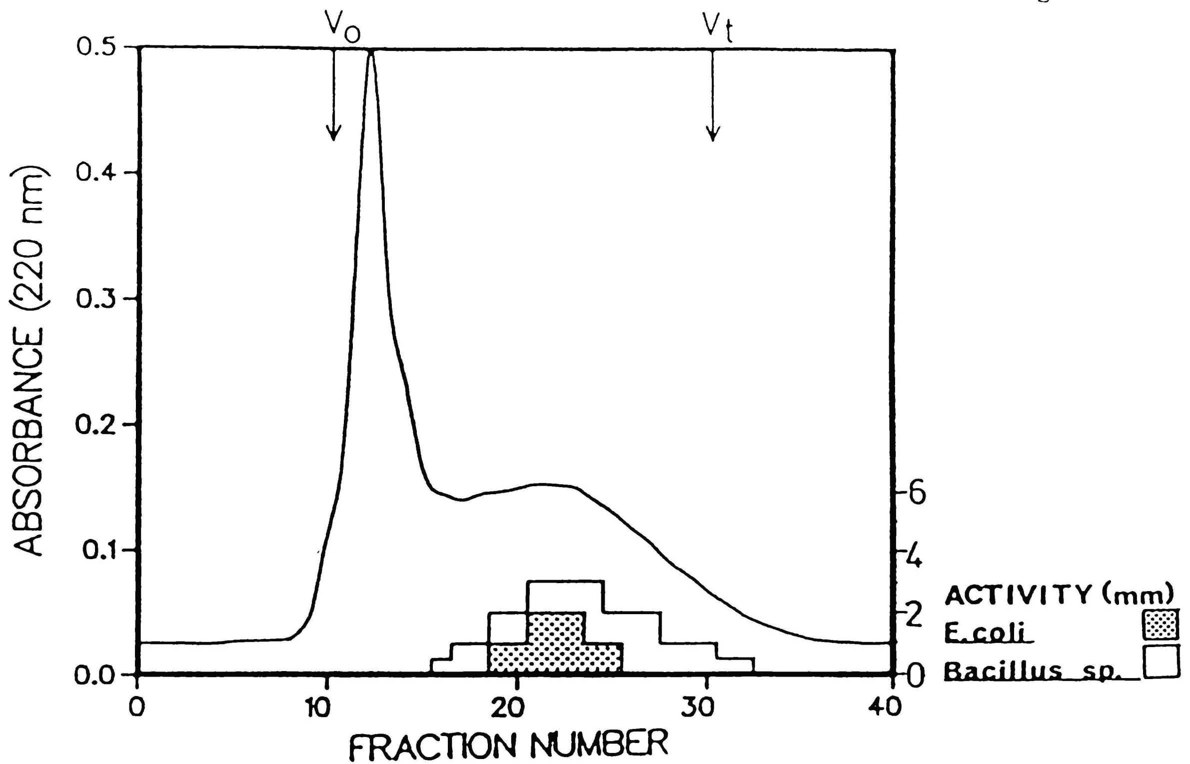


Fig. 5-8a: Gel Filtration Chromatography of the Remainder of Fractions 4-7 from Fig. 5-3
Gel: Sephadex G 75 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 4-7 (Fig. 5-3), desalted, freeze-dried and re-dissolved in 5 ml of 0.02 mol/l HCl, pH 1.7
Fraction Volume: 10 ml

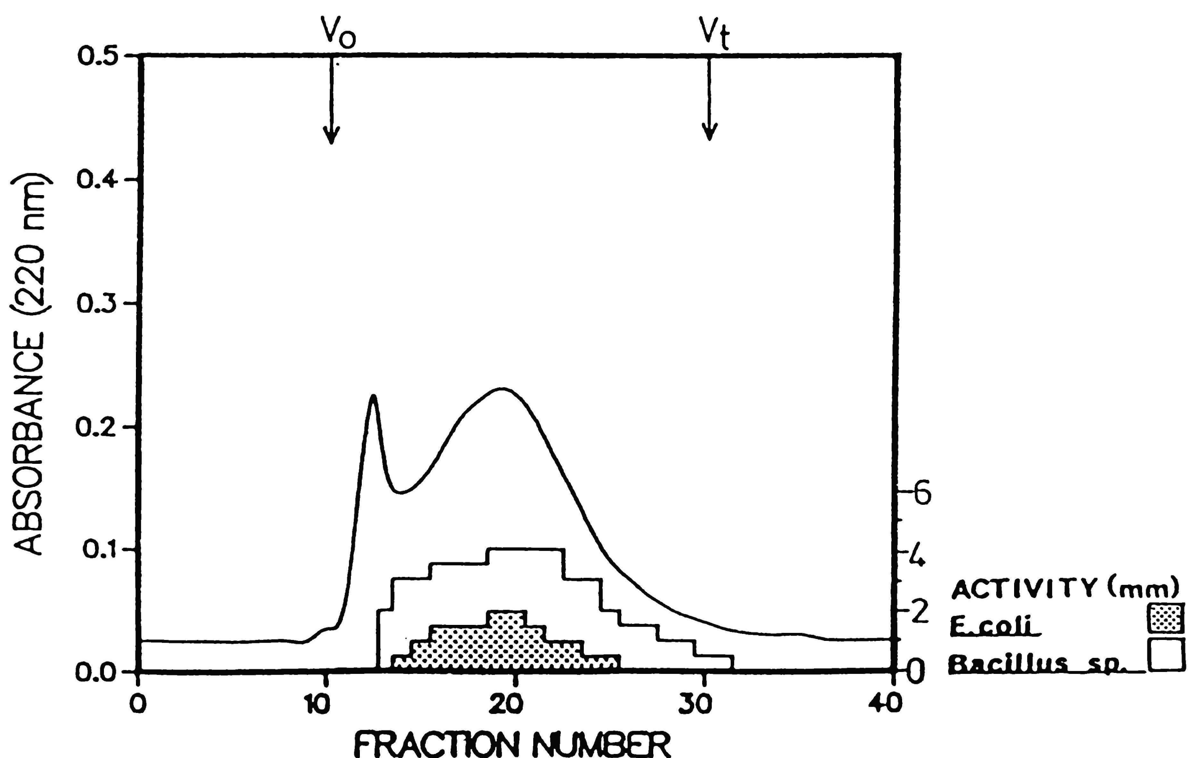


Fig 5-8b: Gel Filtration Chromatography of the Remainder of Fractions 18-34 from Fig. 5-3
Gel: Sephadex G 75 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 18-34 (Fig.5-3), desalted, freeze-dried and re-dissolved in 5 ml of 0.02 mol/l HCl, pH 1.7
Fraction Volume: 10 ml

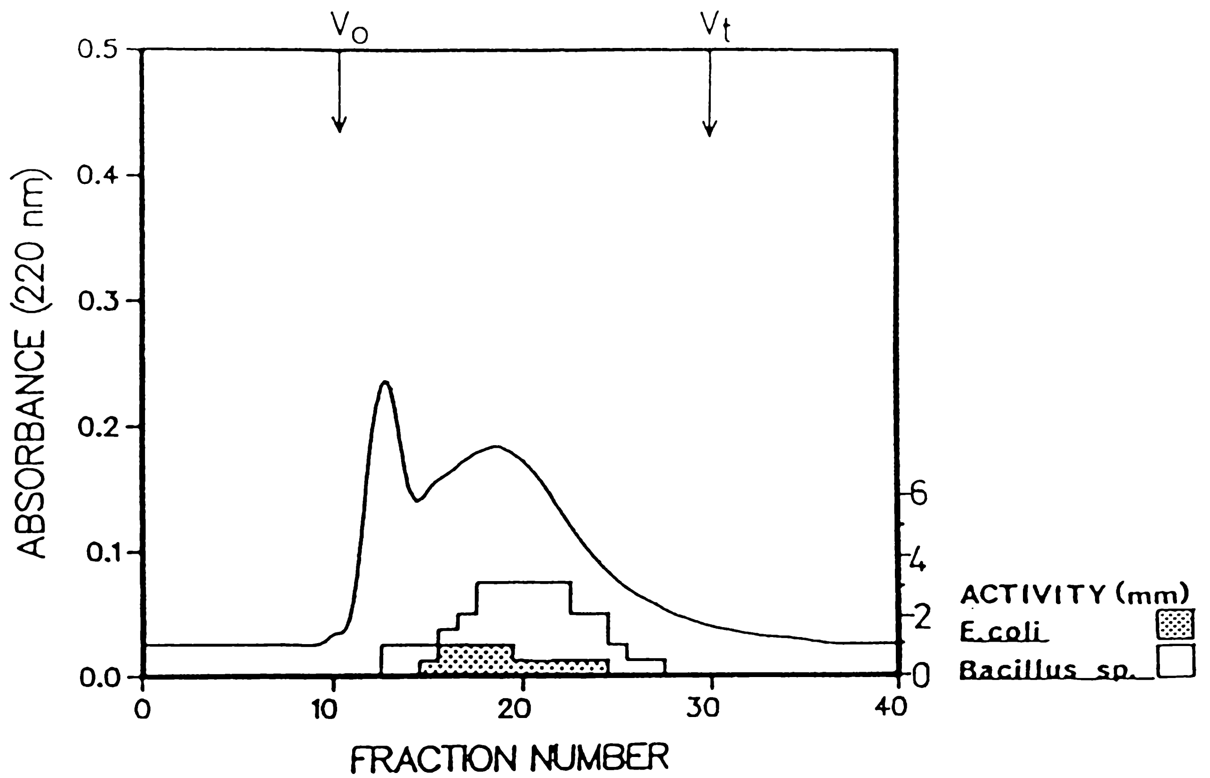


Fig. 5-8c: Gel Filtration Chromatography of the Remainder of Fractions 38-48 from Fig. 5-3
Gel: Sephadex G 75 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 38-48 (Fig. 5-3), desalted, freeze-dried and re-dissolved in 5 ml of 0.02 mol/l HCl, pH 1.7
Fraction Volume: 10 ml

Therefore, gel filtration chromatography in the presence of SDS and 2-mercaptoethanol was used in an attempt to dissociate the active components from other proteins present. As SDS has antibacterial properties, it was removed prior to testing by passing the protein-SDS solutions through an anion exchanger which selectively binds the SDS. The 2-mercaptoethanol was removed by a second ion exchange step using a cation exchanger, to which the protein was bound and subsequently eluted by high pH.

The sample (5 ml of the milk cell extract) was incubated overnight at 37°C with 1% SDS and 1% 2-mercaptoethanol in 0.1 mol/l Tris buffer, pH 7.2. The mixture was fractionated on a column of Sepharose CL-6B (This gel was used because of the increase in molecular size of the SDS-coated proteins) with 0.1% SDS in 0.1 mol/l Tris buffer, pH 7.2, as the eluent. The elution profile showed 4 protein peaks followed by a large peak containing 2-mercaptoethanol (see Fig. 5-9). To examine the degree of separation being achieved samples (1 ml) were taken from the marked fractions throughout the elution profile (fractions 12, 17, 21, 23, 25, 28, 32, 35 and 38). They were freeze-dried and re-dissolved in 100 µl of SDS sample buffer and 20 µl samples were separated by SDS electrophoresis. It was evident from the SDS electrophoresis results shown in Plate 5-3 that the gel filtration chromatography in the presence of 0.1% SDS was not successful, as each fraction was multi-banded with many bands common to each fraction although in different quantities. The fractions making up each of the peaks were pooled and , after raising the pH to 8.9, the SDS was removed by passage through a column of QAE Sephadex A 25, equilibrated with Tris buffer (0.1 mol/l), pH 8.9. The SDS-free protein solution was diluted by adding an equal volume of phosphate buffer (0.01 mol/l), pH 6, and,

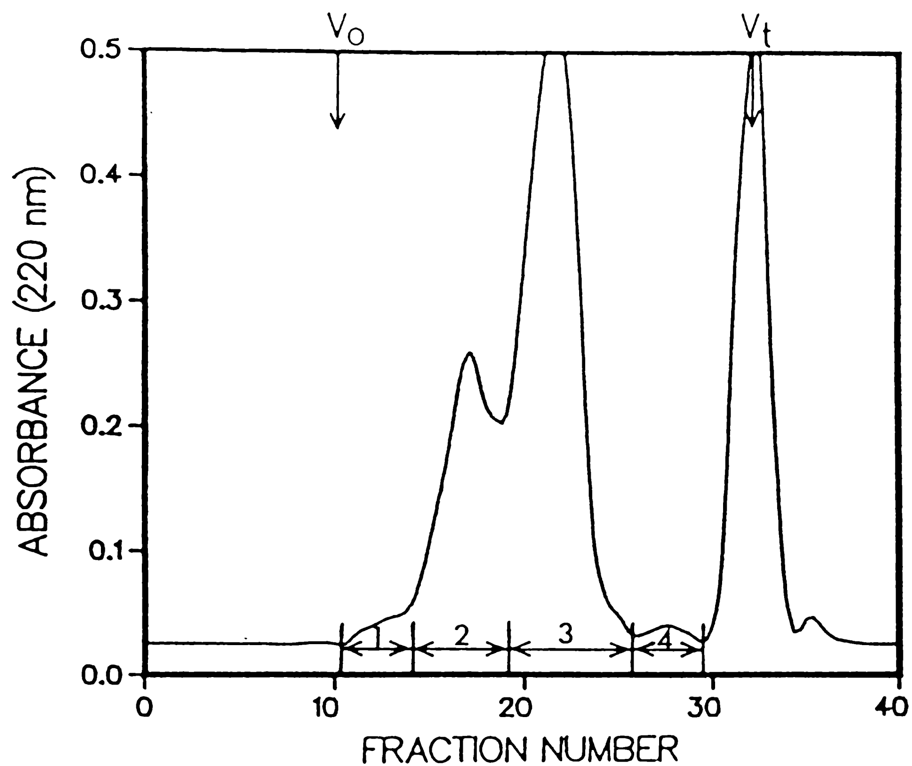


Fig 5-9: Gel Filtration Chromatography of the Milk Cell Extract
Gel: Sepharose CL-6B
Eluent: 0.1 mol/l Tris buffer, pH 7.2 containing 0.1% SDS
Sample: Milk cell extract dissolved in 5 ml of 0.1 mol/l Tris buffer, pH 7.2, containing 1% SDS and 1% 2-mercapoethanol and incubated overnight
Fraction Volume: 10 ml

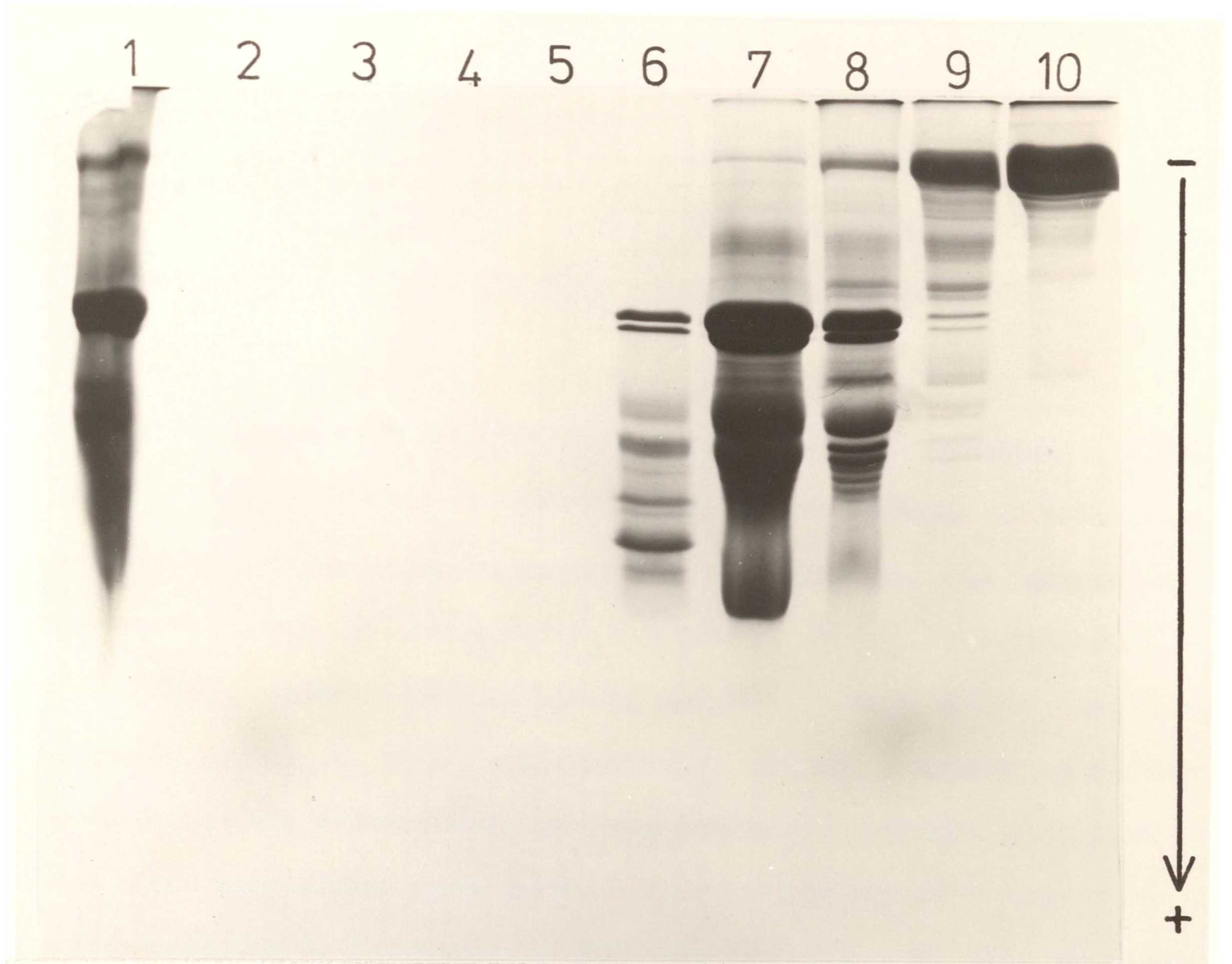


Plate 5-3: SDS Electrophoresis

1 - milk cell extract

2 - fraction 38 from Fig. 5-9

3 - fraction 35 from Fig. 5-9

4 - fraction 32 from Fig. 5-9

5 - fraction 28 from Fig. 5-9

6 - fraction 25 from Fig. 5-9

7 - fraction 23 from Fig. 5-9

8 - fraction 21 from Fig. 5-9

9 - fraction 17 from Fig. 5-9

10- fraction 12 from Fig. 5-9

after adjusting to pH 6, separated from the mercaptoethanol and concentrated by adsorption on a column of SP Sephadex C 25. The cationic material was finally eluted by high pH. Only peak 3 exhibited antibacterial activity but was shown by SDS electrophoresis to be a very complex mixture.

In further attempts to dissociate these complexes, the concentration of SDS in the sample was increased in steps up to a final concentration of 10%. In conjunction with increasing the sample SDS concentration, the SDS concentration in the eluent was also increased to 0.5% with no effect. The 2-mercaptoethanol concentration was also increased up to a final concentration of 5%. However no further separation of the protein complexes was obtained. The sample incubation times and conditions were also varied. Samples were boiled for 2 minutes followed by incubation at 37°C for 24 hours. In all cases an elution profile similar to that in Fig. 5-9 was obtained with the antibacterial activity only in pool 3. Subsequent SDS electrophoresis of the fractions in pool 3 showed them to be multi-banded.

Conclusions

The milk cell extract appeared to be totally uneffected by the dissociating effects of SDS in conjunction with 2-mercaptoethanol. It is apparent that gel filtration and ion exchange chromatography are unsuitable separation methods under these conditions. The separation of the milk cell extract proteins during analytical electrophoresis suggested that this technique on a preparative scale may be suitable for the isolation of the antibacterial compounds.

5.7 The Use of Preparative Electrophoresis and Isoelectric Focusing in the Separation of the Antibacterial Components

Preparative electrophoresis was initially carried out using the cationic discontinuous polyacrylamide gel method of Reisfeld et al. (1962). This method was chosen because samples are easily dissolved in the buffers used and unlike SDS electrophoresis, few contaminating substances are recovered with the extracted sample after electrophoresis. The gel was prepared as described in section 2.11 except that a single sample well was formed across the top of the gel, leaving one centimetre of gel on either side. The sample well was not across the full width of the gel to prevent the severe distortions which can occur along the sides of the gel during electrophoresis. A 1 ml sample of the milk cell extract (10 mg/ml) was placed in the well and a constant current of 40 mA was applied for 3 hours. After electrophoresis the gel was cut up as shown in Fig. 5-10. One portion was stained to visualise the protein bands, while the major portion was cut into strips. Allowances for the swelling of the gel during staining had to be made when comparing the gel portions. During the final destaining step which used 35% ethanol, the gel was shrunk to its original size. Further comparisons could be made by using R_f values to accurately compare bands on the stained portion with those contained in the gel strips.

As a result of protein overloading, the stained gel showed a large amount of streaking with few distinct bands. Each of the 10 strips were placed in a test tube containing 0.5 ml of 0.1 mol/l phosphate buffer, pH 7. The gel was mashed with a glass rod and allowed to soak for at least 1 hour. The solutions were neutralised by the addition of 1 mol/l NaOH and allowed to stand for a further 10 minutes before the pH was

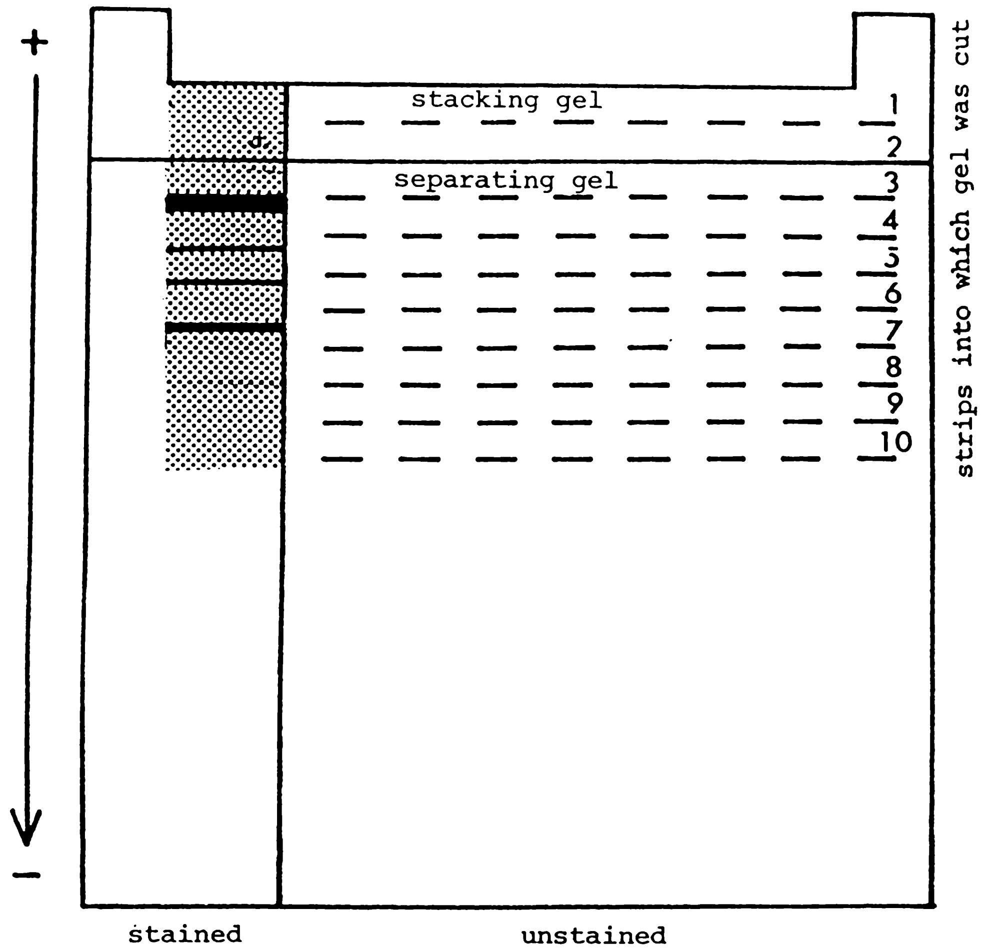


Fig. 5-10: Cutting of Preparative Cationic Electrophoresis Gel

re-checked. Each solution was assayed for antibacterial activity against E. coli and the Bacillus species. Activity was found only in strips 3 and 4, corresponding to a region of marginal penetration into the separating gel (Fig. 5-10). This suggests that the antibacterial compound(s) are of high molecular weight or of low net positive charge at pH 4.5.

In an attempt to obtain better separation of these compounds a gel with a lower acrylamide concentration (10% rather than 15%) was used.

A lysozyme standard was also run to provide a comparison with the mobilities of the antibacterial compounds. The stacking gel was made with a narrow well for the lysozyme standard and a wide well for the milk cell extract. Electrophoresis was carried out at 40 mA for 3 hours, after which the gel was cut into 3 portions as shown in Fig. 5-11. The first portion containing the lysozyme standard and part of the milk cell extract was stained. The central portion was cut into strips and soaked in 0.1 mol/l phosphate buffer, pH 7, and assayed for antibacterial activity. The third portion was cut into strips and soaked in distilled water, and the distilled water extract was freeze-dried.

The antibacterial activity was found only in strip 3 only. However this may have been the only region with a large enough quantity for detection. Other bands present may have been antibacterial if tested at higher concentrations. In the stained portion, strip 3 corresponded to a heavily stained area which probably contained a number of proteins. However, as the antibacterial proteins had still not penetrated far into the separating gel, it was concluded that little change in electrophoretic properties was effected by decreasing the acrylamide

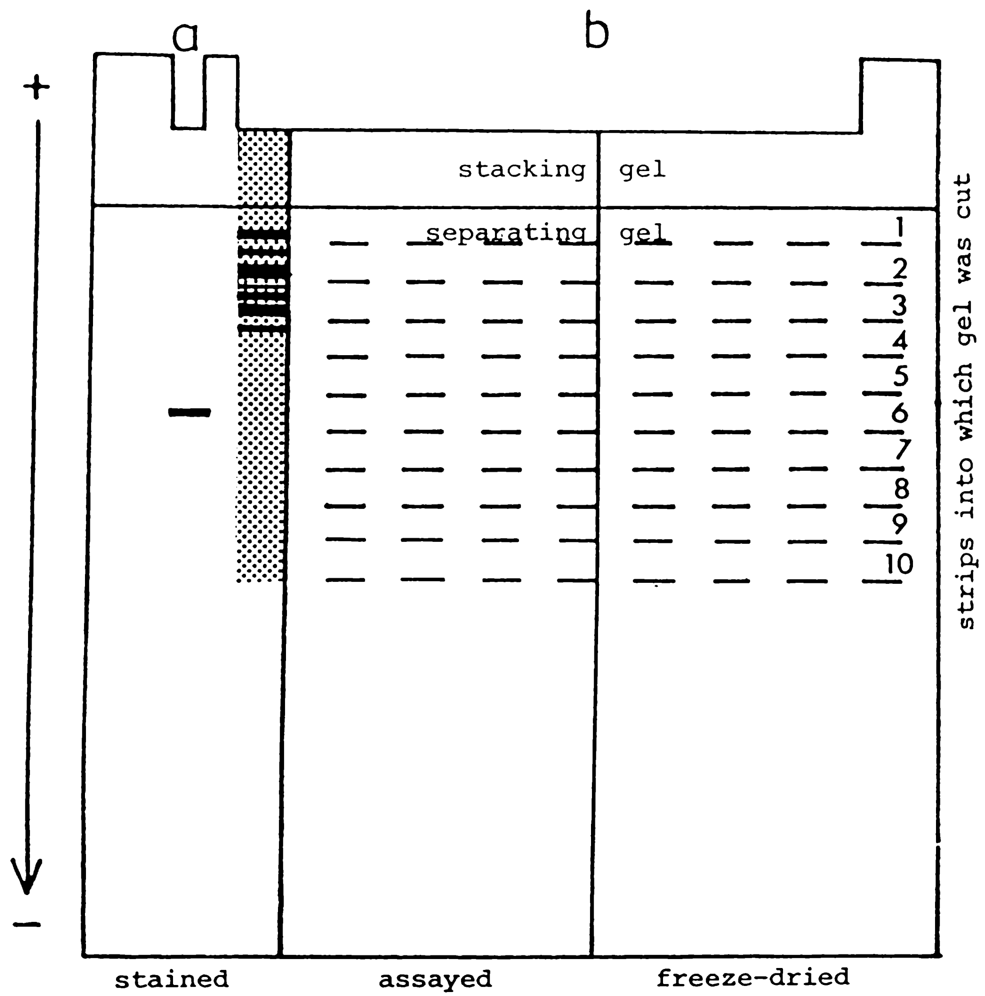


Fig. 5-11: Cutting of Preparative Cationic Electrophoresis Gel.
a- lysozyme standard; b- milk cell extract

concentration. The result supports the suggestion that the antibacterial compounds may not be very cationic at pH 4.5. In comparison lysozyme demonstrated high mobility in the gel. There was no distinct corresponding band in the milk cell extract which further supports the absence of lysozyme. Bovine lysozyme has a similar mobility to egg white lysozyme (Eschenbruch 1980).

The freeze-dried extracts of strips 1 to 6 were separated by SDS electrophoresis. The samples were dissolved in SDS sample buffer and incubated in boiling water for 1 minute. After application of samples to the gel, a 40 mA constant current was applied for 2 hours. The stained gel (Plate 5-4), showed that gel strip 3 (which was antibacterial) contained protein bands similar to those in strips 1 and 2. It was concluded that the antibacterial component(s) might be present as aggregates or bound to the various proteins. It was surprising that apparently identical proteins in strips 1 and 2 were not active. This might be due to the nature of the associated proteins, or the fact that the activity present was below the level of detection of the plate assay method.

Since the separation being achieved by cationic electrophoresis was unsatisfactory, a separation method capable of greater protein dissociation (SDS electrophoresis) was used. Removal of the SDS and 2-mercaptoethanol from electrophoresed samples prior to assaying for antibacterial activity was achieved by dialysis in distilled water for 48 hours using a membrane with a low nominal molecular weight cut-off (3 500).

Plate 5-4: SDS Electrophoresis

1 - strip 6 as shown in Fig. 5-11

2 - strip 5 as shown in Fig. 5-11

3 - strip 4 as shown in Fig. 5-11

4 - strip 3 as shown in Fig. 5-11

5 - strip 2 as shown in Fig. 5-11

6 - strip 1 as shown in Fig. 5-11

7 - milk cell extract

Plate 5-5: SDS Electrophoresis

1 - strip 8 as shown in Fig. 5-12

2 - strip 7 as shown in Fig. 5-12

3 - strip 6 as shown in Fig. 5-12

4 - strip 5 as shown in Fig. 5-12

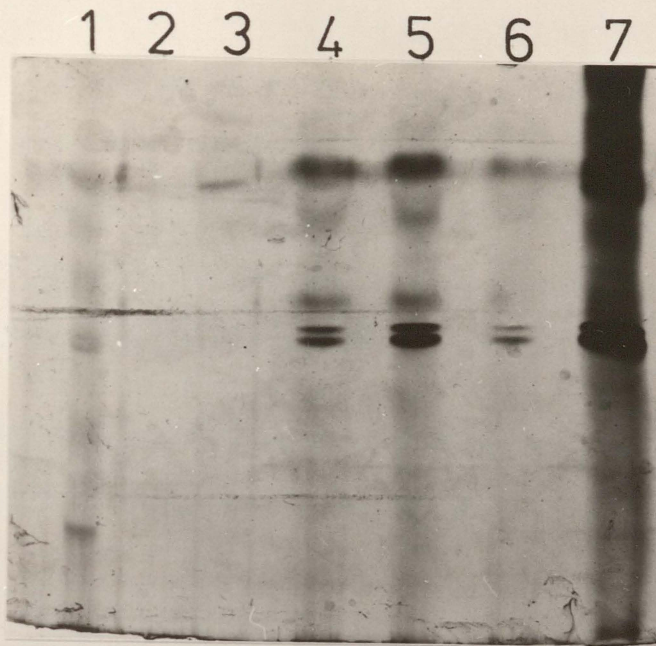
5 - strip 4 as shown in Fig. 5-12

6 - strip 3 as shown in Fig. 5-12

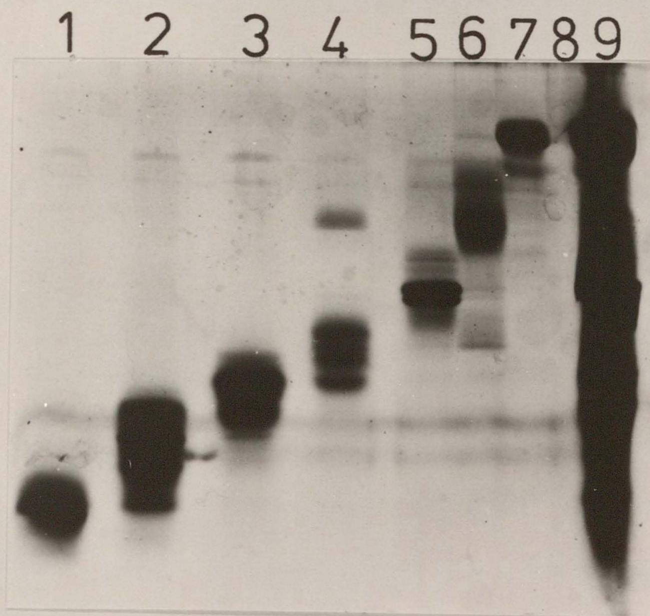
7 - strip 2 as shown in Fig. 5-12

8 - strip 1 as shown in Fig. 5-12

9 - milk cell extract



1 2 3 4 5 6 7
−
+



1 2 3 4 5 6 7 8 9
−
+

Milk cell extract was dissolved in SDS sample buffer containing 1% SDS and 1% 2-mercaptoethanol. This was incubated in a boiling water bath for 2 minutes. The sample (1 ml) was loaded into the horizontal sample well and a current of 40 mA was applied for 2 hours. After electrophoresis one side of the gel was removed for staining and the remaining gel was cut into strips as shown in Fig. 5-12.

The stained portion of the gel was overloaded and did not give a clear indication of the bands present or of the separation being achieved.

Each strip from the unstained portion was mashed in a test tube containing 3 ml of distilled water, and left to soak for at least 1 hour. The extracts were then transferred to low molecular weight cut-off dialysis tubing and dialysed against distilled water for 48 hours at 4°C. The distilled water was changed every 12 hours. In subsequently testing the solutions for antibacterial activity, strips 6 and 7 were found to be positive. Each of the solutions was then freeze-dried. A portion of each freeze-dried sample was re-dissolved in SDS sample buffer and re-run on SDS electrophoresis. The results are shown in Plate 5-5. In the active samples (strips 6 and 7) at least 3 bands were present, of which 1 major band was common to both. It is likely that this band is the antibacterial compound although more than one could be present. It is also interesting to note that strips 6, 7 and 8 have faint bands present in the higher regions of the gel, suggesting that some reaggregation of the lower molecular weight proteins has occurred. Both disaggregation and reaggregation are evident in strips 2, 3, 4 and 5 as there are numbers of bands which have run both well ahead and well behind the molecular weight region from where the strip was originally cut. It is concluded that the treatment

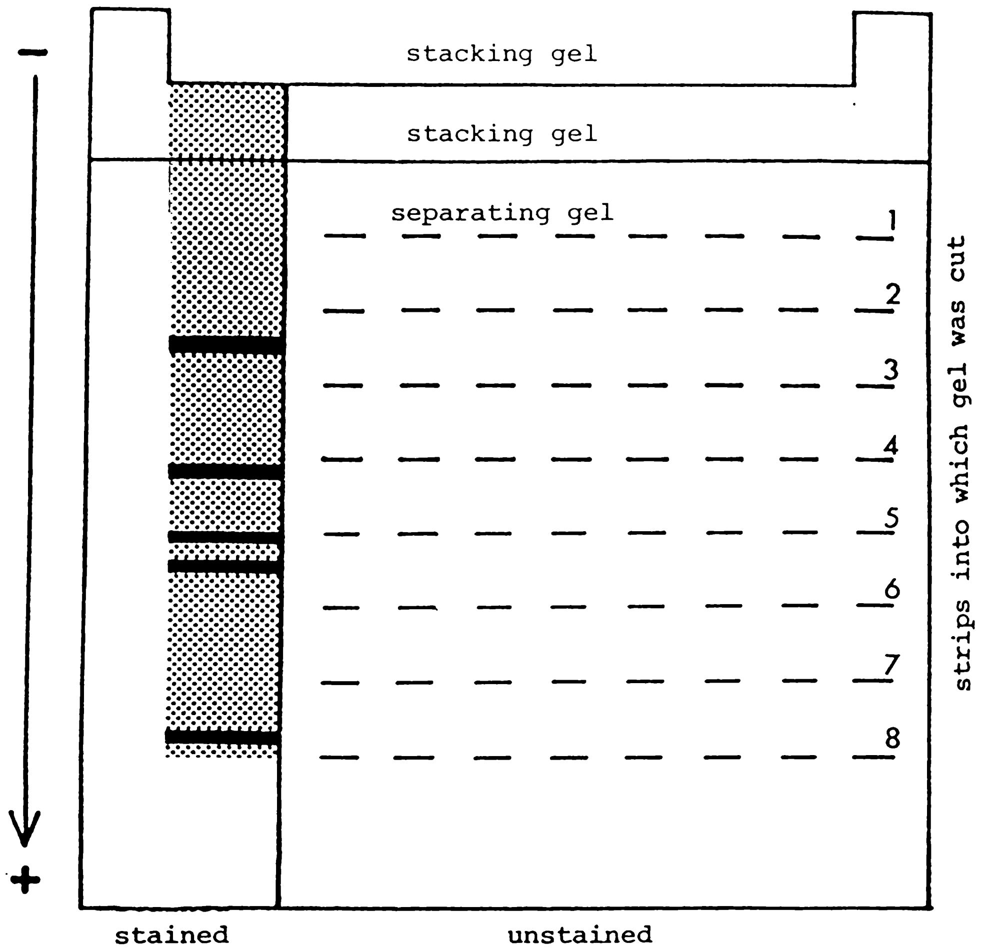


Fig. 5-12: Cutting of Preparative SDS Electrophoresis Gel

of the milk extract with SDS and 2-mercaptoethanol is not completely dissociating the high molecular weight protein complexes.

Owing to the unsatisfactory separation of the milk cell extract on electrophoresis gels, isoelectric focusing was tried. LKB polyacrylamide gel plates which have a focusing range from pH 3.5-9.5, were first used.

The milk cell extract was dissolved in distilled water (1 mg/ml), 10 μl was applied to the polyacrylamide gel, and 5W constant power was applied for 2 hours. After this time period the cytochrome c standard was observed to be focused at the cathode and the ampholine bands were obvious which indicates that they had focused. After staining, the gel showed a series of bands across the gel accompanied by a large amount of streaking (see Plate 5-6), the latter possibly due to the proteins not having reached their isoelectric points. In an attempt to decrease the degree of streaking, isoelectric focusing was carried out at a higher voltage for a short time span, and also with a low voltage for a long time span. It was evident from the results of this that the longer time span decreased the amount of streaking. To determine the mobility of the protein milk cell extract, an isoelectric focusing gel was electrophoresed for 3 hours, during which time identical samples were applied at intervals. The results are shown in Plate 5-7. It is evident that during the course of the isoelectric focusing some bands at the anionic end of the gel (pH 5-6) disappeared with a concomitant increase in protein at the cathodic end (pH 8.5-9.5). This could possibly be caused by the dissociation of basic components from the anionic aggregates, allowing both separated components to travel to their respective isoelectric points.

Plate 5-6: Isoelectric Focusing

1 - myoglobin standard

2 - cytochrome c standard

3 - milk cell extract

4 - milk cell extract

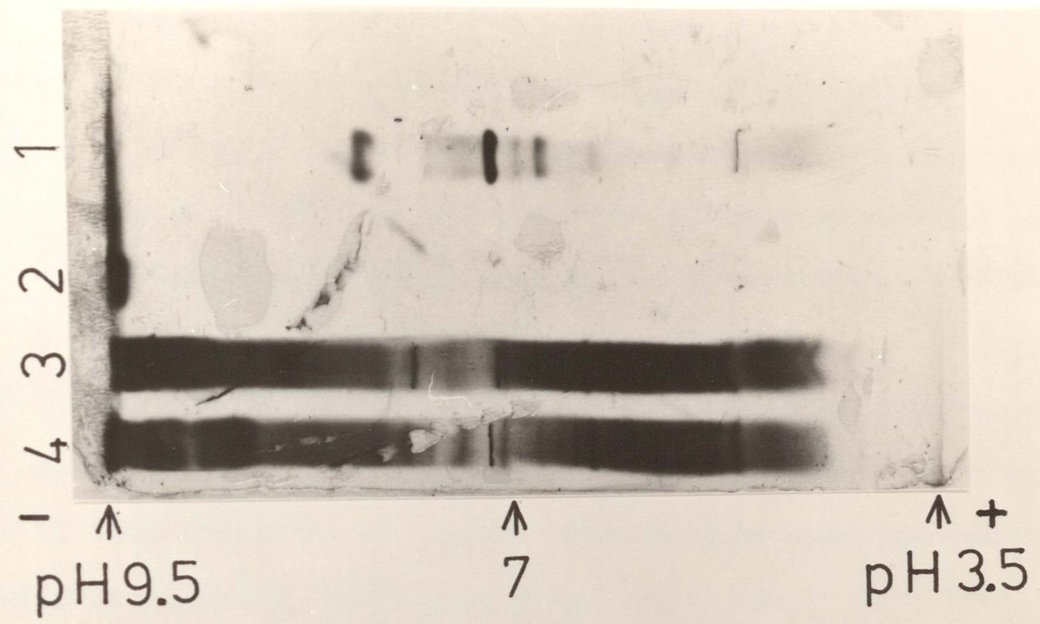
Plate 5-7: Isoelectric Focusing

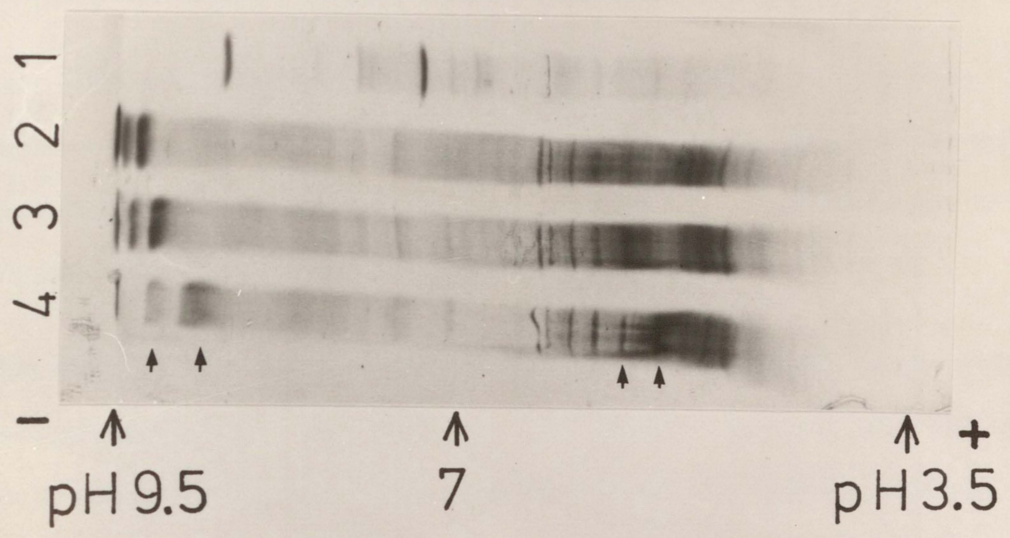
1 - myoglobin standard

2 - milk cell extract focused for 3 hours

3 - milk cell extract focused for 2 hours

4 - milk cell extract focused for 1 hour


1
2
3
4
-
+
pH 9.5
7
pH 3.5


1
2
3
4
-
+
pH 9.5
7
pH 3.5

Although the amount of streaking was reduced there was still some remaining. The end point of the run was hard to determine: if the run is to long cathodic drift results, therefore the cationic proteins near the wick will be lost, whereas if the run is too short then there is not enough time for the components to disaggregate and focus. In a further attempt to reduce streaking and allow complete disaggregation, long runs (15-18 hours) at low voltage were carried out. The distortions normally caused by the presence of carbon dioxide over long periods were diminished by inserting paper wicks soaked in 1 mol/l NaOH into the electrophoresis chamber, and by continuous flushing with oxygen-free nitrogen. Despite these precautions, severe distortions of the gel usually occurred, attributed to either overheating or cathodic drift. This method was deemed most unreliable and was therefore not used on a preparative scale.

In order to investigate the milk cell extract further and to gain an insight into the binding of the antibacterial compounds into the aggregated complexes, the protein milk cell extract was chemically modified by a variety of methods prior to SDS electrophoresis.

The role of disulphide bonds in the milk cell extract was first examined. If disulphide bonds were important in protein aggregation then reduction by 2-mercaptoethanol would be expected to produce significant changes in the electrophoretic pattern.

Two samples of the milk cell extract were prepared, one with 1% 2-mercaptoethanol and 1% SDS in the sample buffer, and the second without 2-mercaptoethanol. Portions of each of these samples were examined by SDS electrophoresis with a constant current of 40 mA, for 2-3 hours. No significant differences were seen in the stained gel

containing samples with and without 2-mercaptoethanol (see Plate 5-8). It was concluded that either disulphide bonds were of little importance in the association of the protein complexes, or that 2-mercaptoethanol was not reducing the bonds sufficiently. A more effective reducing agent, dithiothreitol (Cleland, 1964) was used in place of 2-mercaptoethanol. A sample of the milk cell extract was treated with a sample buffer containing 20 mmol/l dithiothreitol and 1% SDS. This and a control without dithiothreitol were then run on SDS electrophoresis. The resulting electrophoretic pattern (see Plate 5-8) showed that little change had taken place. To eliminate the possibility that reduced disulphide bonds were re-forming during electrophoresis, the samples were reduced and alkylated to prevent disulphide bond reformation. Urea was also added as a protein denaturent, to ensure that all disulphide bonds were accessible.

Samples of the milk cell extract were reduced with 2-mercaptoethanol and alkylated using a modification of the method of Weil and Seible (1961). The extract (10 mg) was added to 17 µl of 2-mercaptoethanol in 180 µl of 9 mol/l urea (pH approximately 8.5). This was incubated overnight at 37°C in a sealed tube under a nitrogen atmosphere. Acrylonitrile (27 µl) was added and left for approximately 4 hours until the odour had disappeared. Finally, SDS was added (2% w/v) and the pH adjusted to 6.8. The sample was separated by SDS electrophoresis as shown in Plates 5-8 and 5-9. The most significant change resulting from this treatment was in the area of high molecular weight where two bands migrated together (see arrow). In the treated sample the top band was no longer present. In addition, two rapidly migrating bands (close to the dye front) appeared. These were presumably small peptides or artifacts.

Plate 5-8: SDS Electrophoresis

1 - lysozyme standard

2 - cytochrome c standard

3 - not relevant

4 - not relevant

5 - not relevant

6 - not relevant

7 - milk cell extract treated with SDS and 2-mercaptoethanol

8 - milk cell extract treated with SDS and 2-mercaptoethanol

9 - milk cell extract treated with SDS and no disulphide-reducing agent

10- milk cell extract treated with SDS and no disulphide-reducing agent

Plate 5-9: SDS Electrophoresis

1 - cytochrome c standard

2 - lysozyme standard

3 - milk cell extract treated with no disulphide-reducing agent

4 - milk cell extract treated with SDS and 2-mercaptoethanol

5 - milk cell extract treated with SDS and dithiothreitol

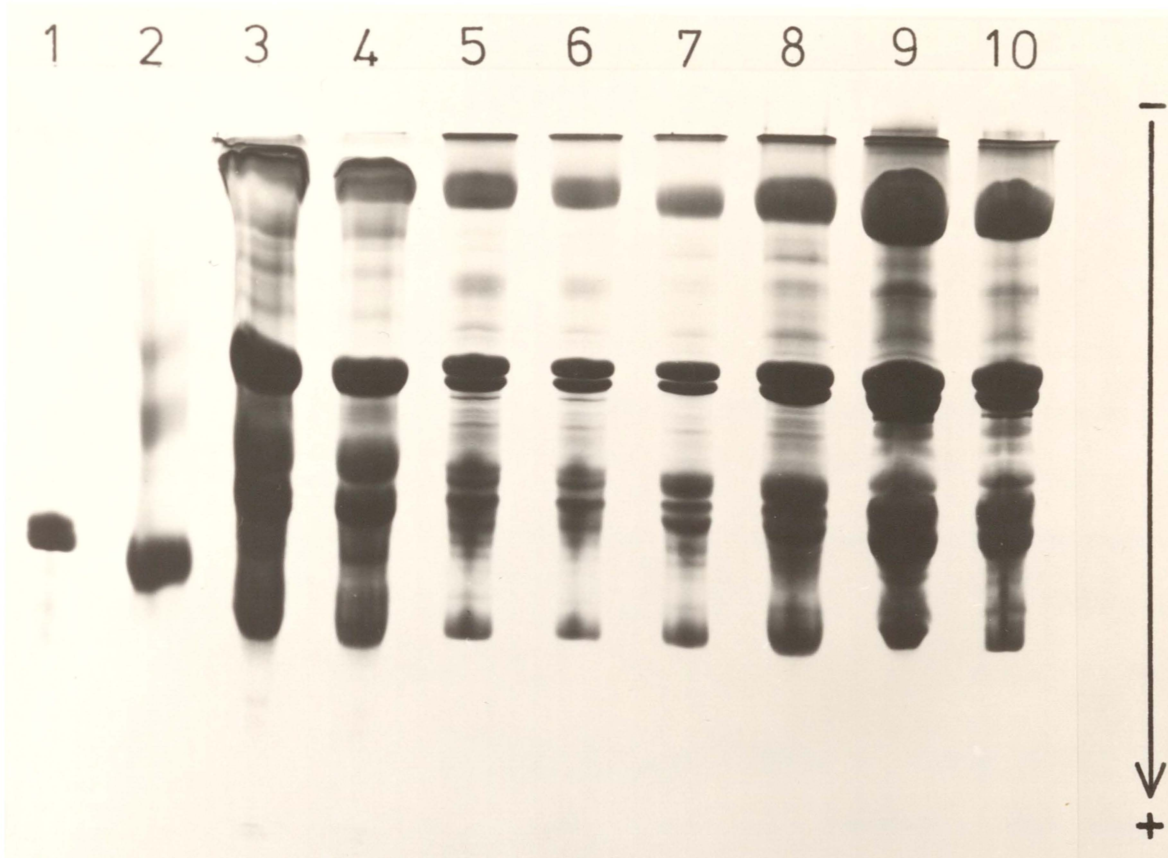
6 - milk cell extract treated with SDS and dithiothreitol

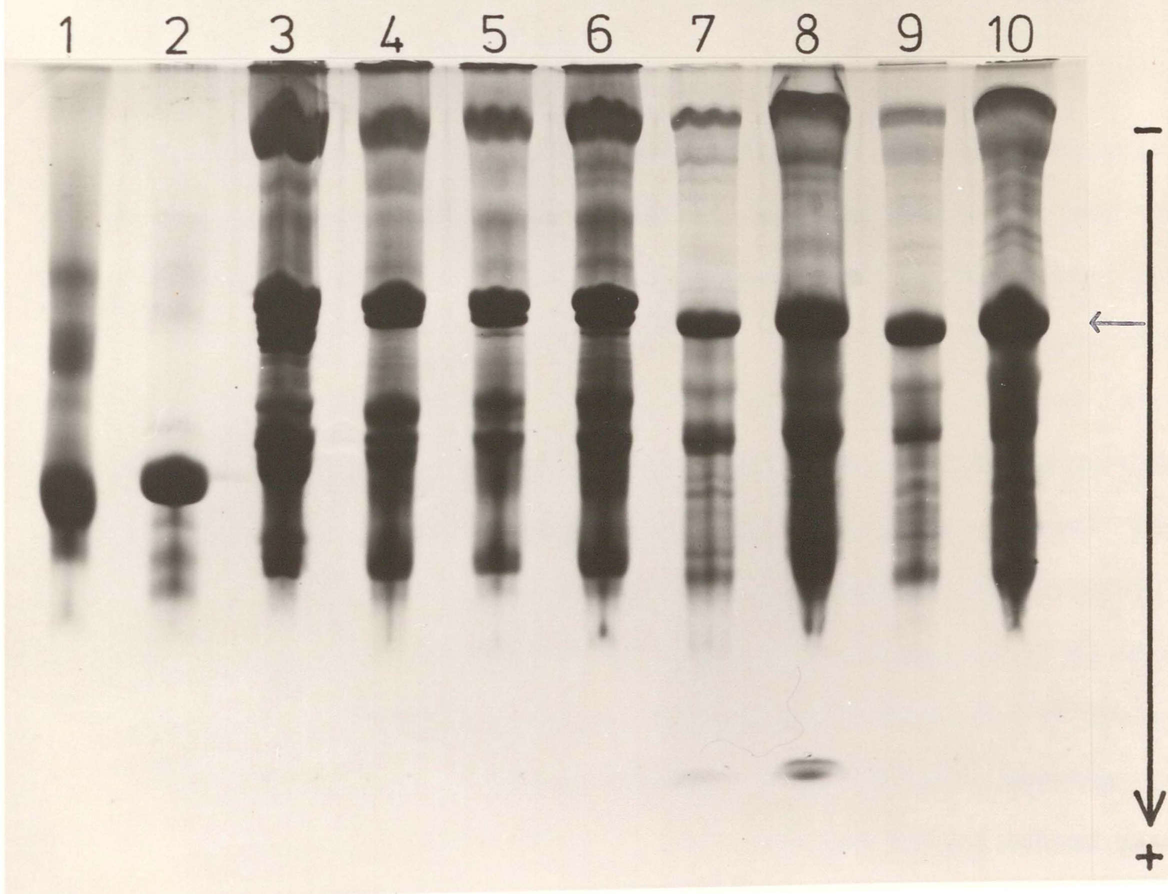
7 - milk cell extract treated with SDS, 9 mol/l urea, mercaptoethanol and acrylonitrile

8 - milk cell extract treated with SDS, 9 mol/l urea, mercaptoethanol and acrylonitrile

9 - milk cell extract treated with SDS, 9 mol/l urea, dithiothreitol and acrylonitrile

10- milk cell extract treated with SDS, 9 mol/l urea, dithiothreitol and acrylonitrile


1
2
3
4
5
6
7
8
9
10
−
+


1
2
3
4
5
6
7
8
9
10
−
+

Another sample of the milk cell extract was treated as above, but with dithiothreitol instead of 2-mercaptoethanol. This was run on SDS electrophoresis and a similar pattern emerged with the high molecular weight bands (see Plate 5-9). However no fast migrating bands were present on the dye front. This suggests that these bands might be artifacts, possibly a product of 2-mercaptoethanol and acrylonitrile. A sample containing these reagents without protein present was prepared to test this possibility in the next run. In an effort to further reduce the amount of protein streaking, the concentration of SDS in the gels was raised from 0.1% to 0.5%.

Samples were run on the 0.5% SDS gel as shown in Plate 5-10, but showed little difference in the amount of steaking. The sample containing only 2-mercaptoethanol and acrylonitrile did not give bands of low molecular weight on the dye front. It is therefore likely that these bands are a result of the action of the reagents on the milk cell extract, disaggregating a small peptide.

This peptide may have been disaggregated from the antibacterial component(s) in the milk cell extract, and therefore it may be antibacterial, and warrant further investigation. This possibility was checked by extracting the active area from a preparative SDS electrophoresis gel as outlined earlier, and the extract treated with the 2-mercaptoethanol and acrylonitrile reagents to see if the peptide resulted from the antibacterial activity. The milk cell extract was prepared using the standard method (1% SDS and 1% 2-mercaptoethanol). This was applied and run for 2 hours at a constant current of 40 mA on a preparative SDS electrophoresis gel. The gel was sliced into strips as outlined above in Fig. 5-12. After mashing the strips and soaking in distilled water for 3-4 hours, extracts were dialysed (using tubing with

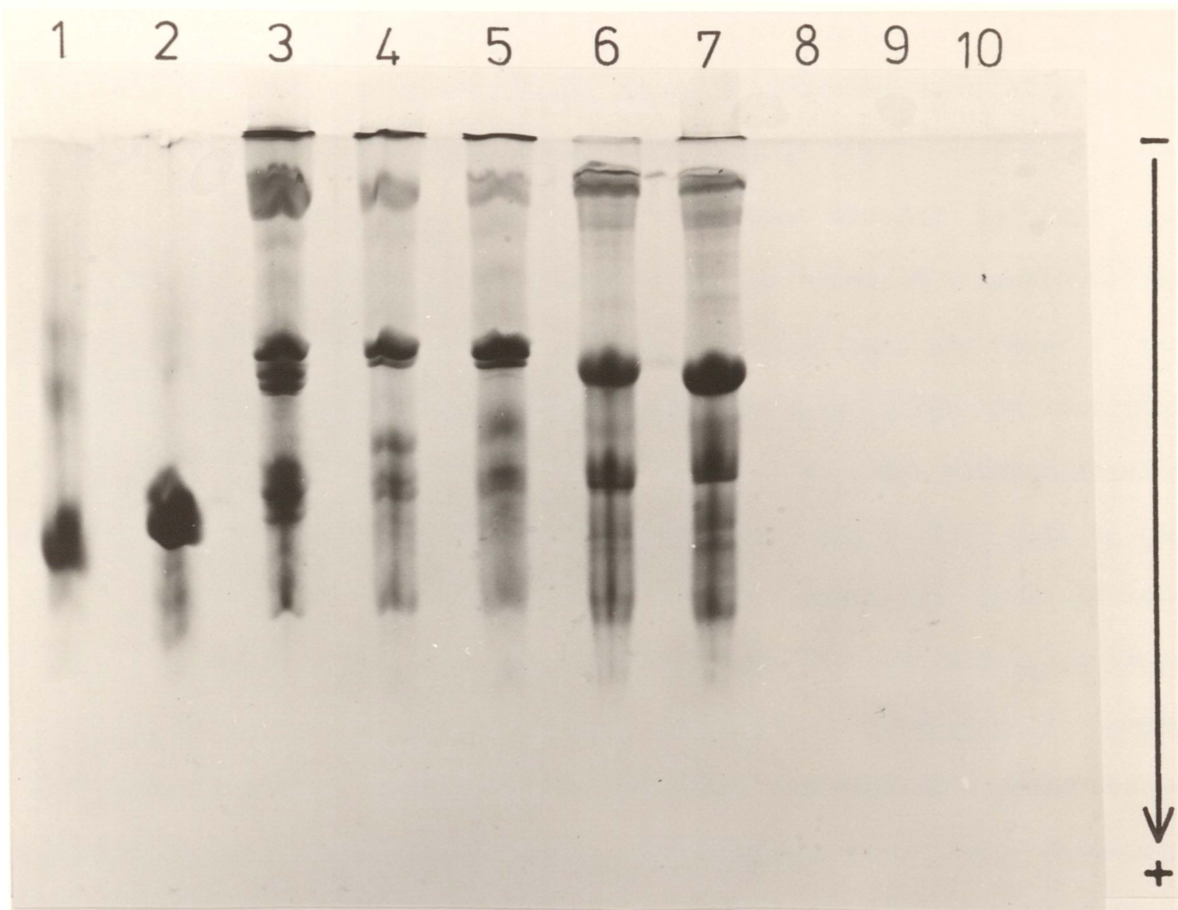


Plate 5-10: SDS Electrophoresis

1 - cytochrome c standard

2 - lysozyme standard

3 - milk cell extract treated with SDS and no disulphide reducing agent

4 - milk cell extract treated with SDS and 2-mercaptoethanol

5 - milk cell extract treated with SDS and dithiothreitol

6 - milk cell extract treated with SDS, 9 mol/l urea, mercaptoethanol and acrylonitrile

7 - milk cell extract treated with SDS, 9 mol/l urea, dithiothreitol and acrylonitrile

8 - SDS, 9 mol/l urea, mercaptoethanol and acrylonitrile standard

9 - spermine standard

10- spermine standard

a cut-off of molecular weight 3 500) against distilled water at 4°C for 48 hours. Antibacterial activity was detected in fractions 6-8 with the majority in fraction 7. Each fraction was divided in half and freeze-dried. One half was treated with the normal SDS sample buffer (1% SDS and 1% 2-mercaptoethanol), while the other half was reduced and alkylated with 2-mercaptoethanol and acrylonitrile as outlined previously. These samples were run in parallel on SDS electrophoresis gels. The results are shown in Plate 5-11 and 5-12). None of the samples treated with mercaptoethanol and acrylonitrile gave protein bands on the dye front. It was concluded that the fast-migrating bands were unrelated to the antibacterial components. Fraction 7, which contained the most antibacterial activity, occurred as a broad region rather than one or more sharp bands. Explanations for this behaviour might be that more than one protein was present, or that the SDS-protein binding interactions were modified by the cationic nature of the protein. This could result in variable coatings of SDS and hence might generate considerable heterogeneity in protein mobility.

Reduction and alkylation of fraction 6 resulted in the loss of one of the two major bands but produced no proteins of lower molecular weight. This fraction had only slight antibacterial activity which may have been picked up from fraction 7. Fraction 8 also had a small amount of antibacterial activity, which could also have been picked up from fraction 7.

Also of interest, as was noticed earlied, is that the two bands present which migrated close together in fraction 3, when reduced and alkylated formed into 1 major band of slightly greater mobility. These bands were not antibacterial and did not yield any more highly mobile proteins on alkylation, which may have been active.

Plate 5-11: SDS Electrophoresis

1 - milk cell extract treated with SDS and 2-mercaptoethanol

2 - strip 1 (Fig. 5-12) treated with SDS and 2-mercaptoethanol

3 - strip 1 (Fig. 5-12) treated with SDS, 2-mercaptoethanol and acrylonitrile

4 - strip 2 (Fig. 5-12) treated with SDS and 2-mercaptoethanol

5 - strip 3 (Fig. 5-12) treated with SDS and 2-mercaptoethanol

6 - strip 3 (Fig. 5-12) treated with SDS, 2-mercaptoethanol and acrylonitrile

7 - strip 4 (Fig. 5-12) treated with SDS and 2-mercaptoethanol

8 - strip 4 (Fig. 5-12) treated with SDS, 2-mercaptoethanol and acrylonitrile

9 - strip 5 (Fig. 5-12) treated with SDS and 2-mercaptoethanol

10- strip 5 (Fig. 5-12) treated with SDS, 2-mercaptoethanol and acrylonitrile

Plate 5-12: SDS Electrophoresis

1 - milk cell extract treated with SDS and 2-mercaptoethanol

2 - strip 6 (Fig. 5-12) treated with SDS and 2-mercaptoethanol

3 - strip 6 (Fig. 5-12) treated with SDS, 2-mercaptoethanol and acrylonitrile

4 - strip 7 (Fig. 5-12) treated with SDS and 2-mercaptoethanol

5 - strip 7 (Fig. 5-12) treated with SDS, 2-mercaptoethanol and acrylonitrile

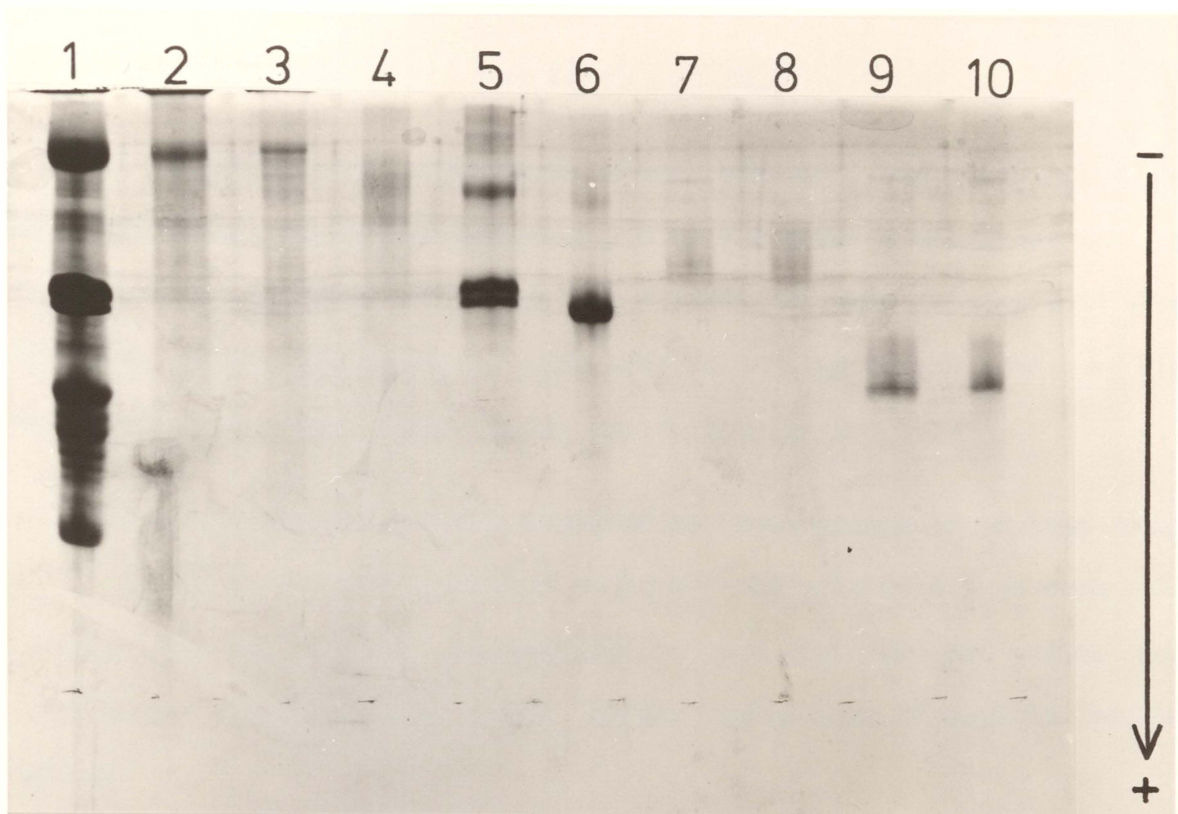
6 - strip 8 (Fig. 5-12) treated with SDS and 2-mercaptoethanol

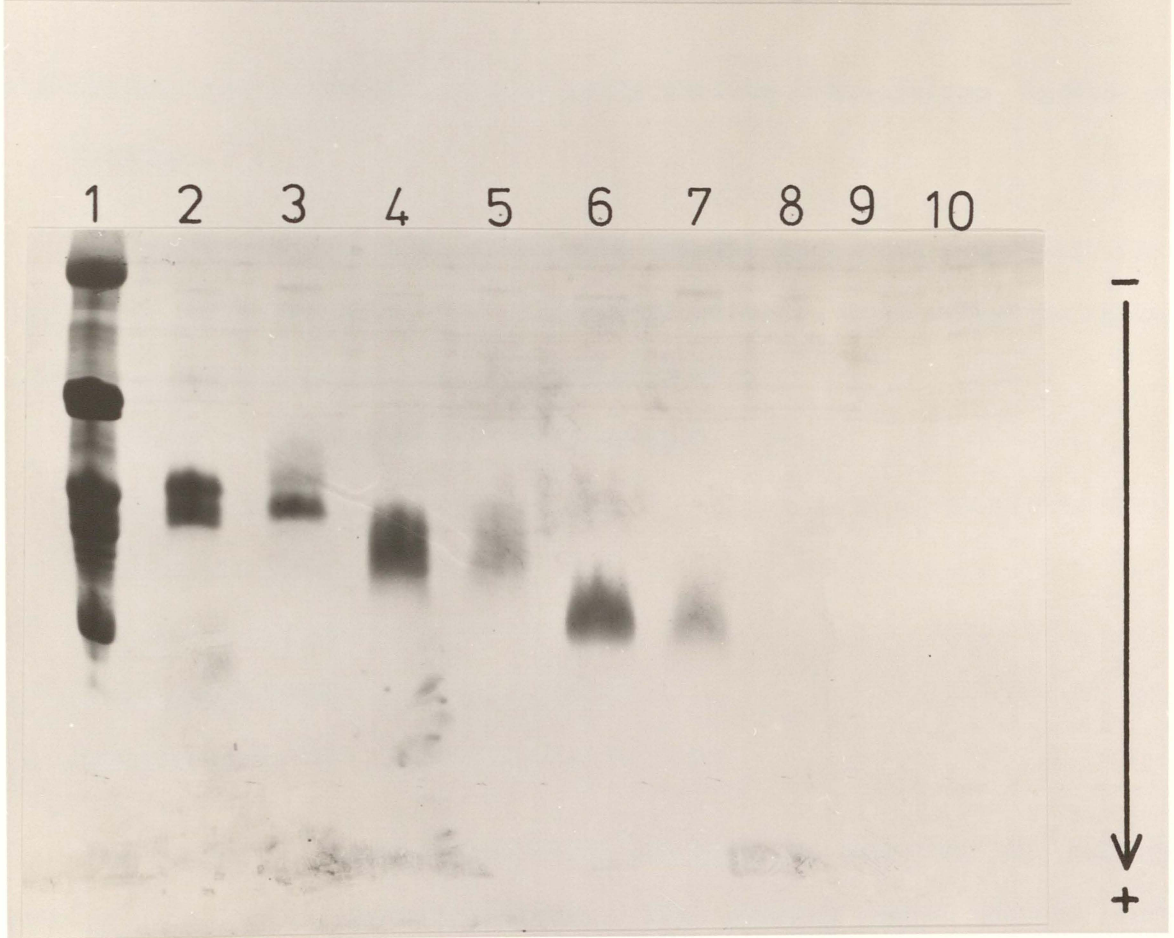
7 - strip 8 (Fig. 5-12) treated with SDS, 2-mercaptoethanol and acrylonitrile

8 - strip 9 (Fig. 5-12) treated with SDS and 2-mercaptoethanol

9 - strip 9 (Fig. 5-12) treated with SDS, 2-mercaptoethanol and acrylonitrile

10- 2-mercaptoethanol and acrylonitrile standard


1
2
3
4
5
6
7
8
9
10
−
+


1
2
3
4
5
6
7
8
9
10
−
+

Conclusions

It appears that disulphide bonds are not important in the aggregation of the active compound(s) with the other proteins present in the milk cell extract. Although some alterations were noticed with some proteins after alkylation, these were not in the active area, nor did they yield additional bands in the active area of the gel.

A major disadvantage with preparative electrophoresis is that only small amounts of antibacterial compound are available from each gel. Further work on protein disaggregation was carried out using gel filtration chromatography where larger quantities of material can be applied.

5.8 Gel Filtration Chromatography using various Dissociating Agents as Eluents

In further attempts to dissociate the antibacterial compounds from the milk cell extract the following eluents were tested using gel filtration chromatography with Sephadex G 75 F:

various concentrations of NaCl up to 4.0 mol/l

6 mol/l urea

10% formic acid

0.1 mol/l formamide

0.01 mol/l NaOH

1% triton X 100

The degree of dissociation being achieved was measured by running samples on SDS electrophoresis. The eluents were removed from chromatography fractions by dialysis against water using tubing of low molecular weight cut-off (nominal limit of 3 500), prior to assaying for

antibacterial activity and then freeze-drying.

All the eluents listed above gave broad elution profiles on Sephadex G 75 and the active fractions gave multibanded patterns when separated by SDS electrophoresis (similar to that obtained in Plate 5-3 from gel filtration chromatography with 0.1% SDS in the eluent).

Ethylenediaminetetraacetic acid (EDTA) was also tried as a dissociating agent. It was hoped that the cationic antibacterial compounds might bind preferentially to the anionic EDTA thus promoting disaggregation.

Milk cell extract (50 mg) was dissolved in 5 ml of 0.01 mol/l EDTA. It was allowed to stand at room temperature for approximately one hour before being chromatographed on a column of Sephadex G 75 eluted with 0.1 mol/l Tris buffer, pH 7.4. A typically broad-peaked elution profile resulted (see Fig. 5-13), with antibacterial activity being detected throughout. The activity in the bed volume peak was attributed to the EDTA derived from the sample. Samples (1 ml) were taken from throughout the elution profile and freeze-dried. They were then re-dissolved in SDS sample buffer and run on SDS electrophoresis. It was evident from the results shown in Plate 5-13 that some separation in the later fractions was being achieved. Active fractions 24-33 showed very little material of high molecular weight although several bands were still present. These bands also appeared to be in the same position as the active bands from the preparative electrophoresis carried out earlier.

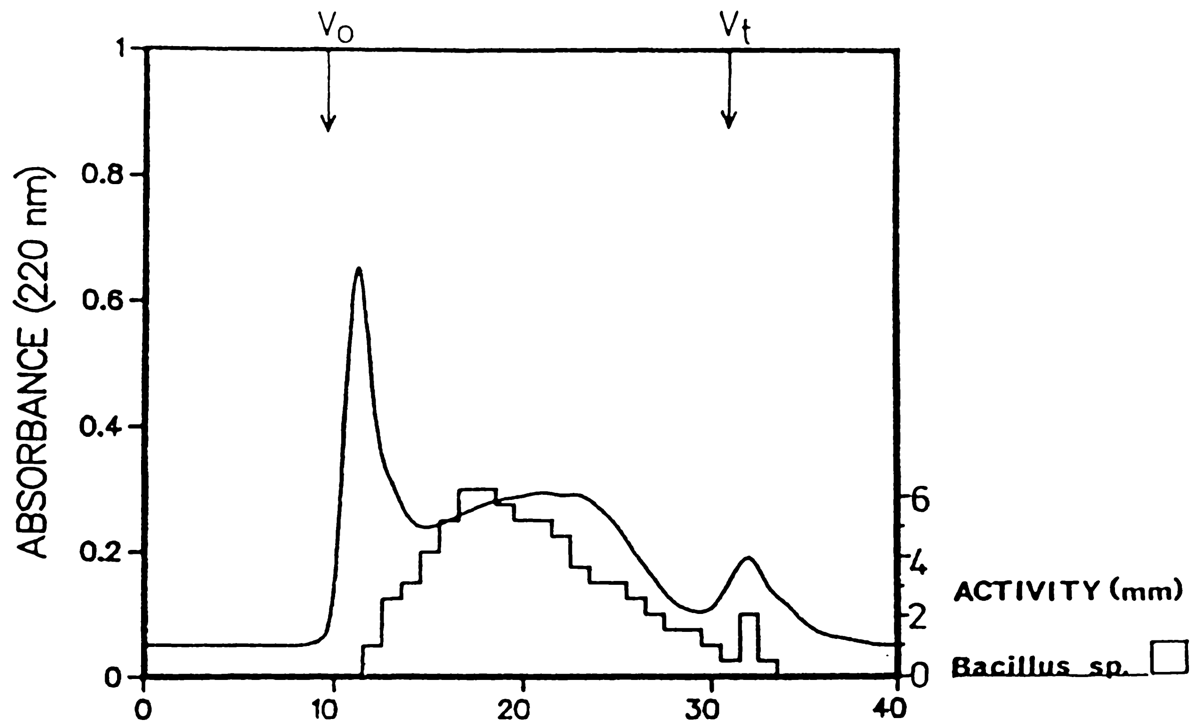


Fig. 5-13: Gel Filtration Chromatography of the Milk Cell Extract
Gel: Sephadex G 75 F
Eluent: 0.1 mol/l Tris buffer, pH 7.4
Sample: Milk cell extract dissolved in 5 ml of 0.01 mol/l EDTA in 0.1 mol/l Tris buffer, pH 7.4
Fraction Volume: 10 ml

Plate 5-13: SDS Electrophoresis

1 - milk cell extract

2 - fraction 11 from Fig. 5-13

3 - fraction 13 from Fig. 5-13

4 - fraction 16 from Fig. 5-13

5 - fraction 18 from Fig. 5-13

6 - fraction 20 from Fig. 5-13

7 - fraction 24 from Fig. 5-13

8 - fraction 26 from Fig. 5-13

9 - fraction 28 from Fig. 5-13

10- fraction 33 from Fig. 5-13

Plate 5-14: SDS Electrophoresis

1 - milk cell extract

2 - fraction 18 from Fig. 5-19

3 - fraction 17 from Fig. 5-19

4 - fraction 16 from Fig. 5-19

5 - not relevant

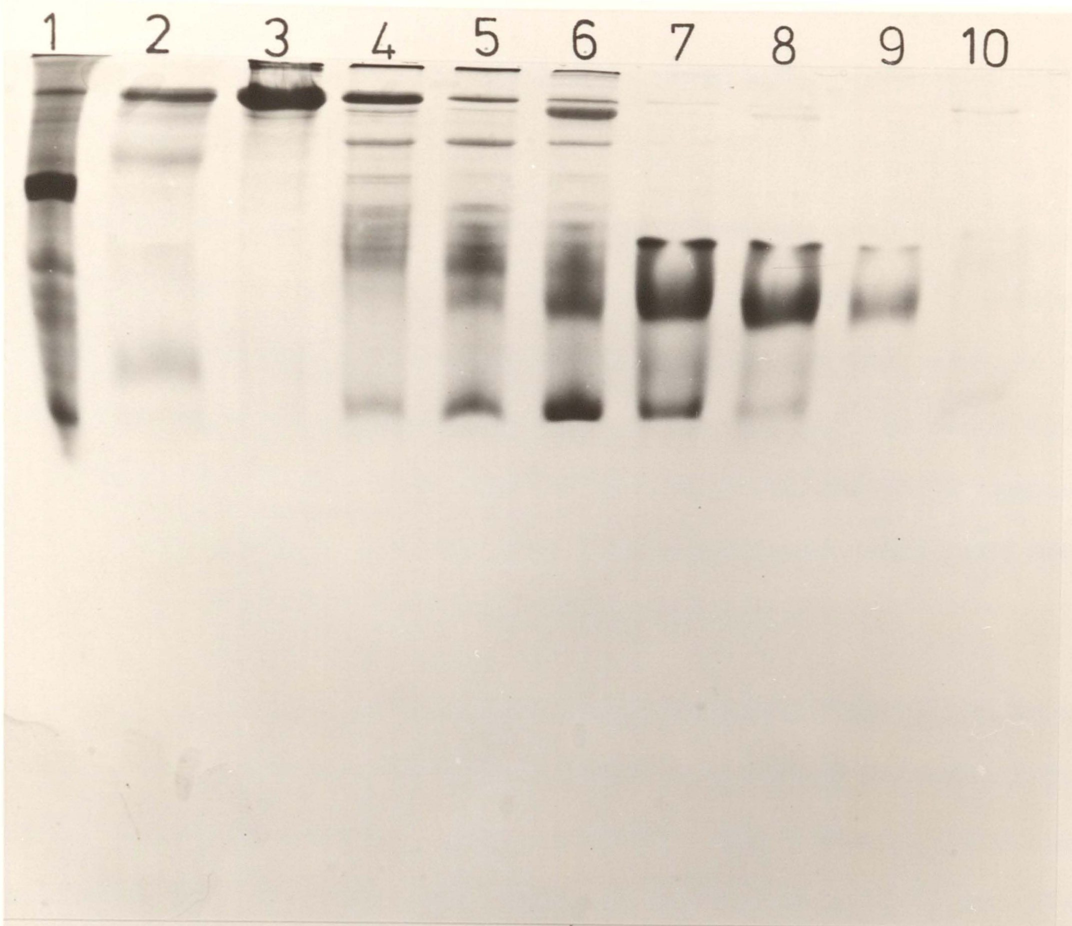
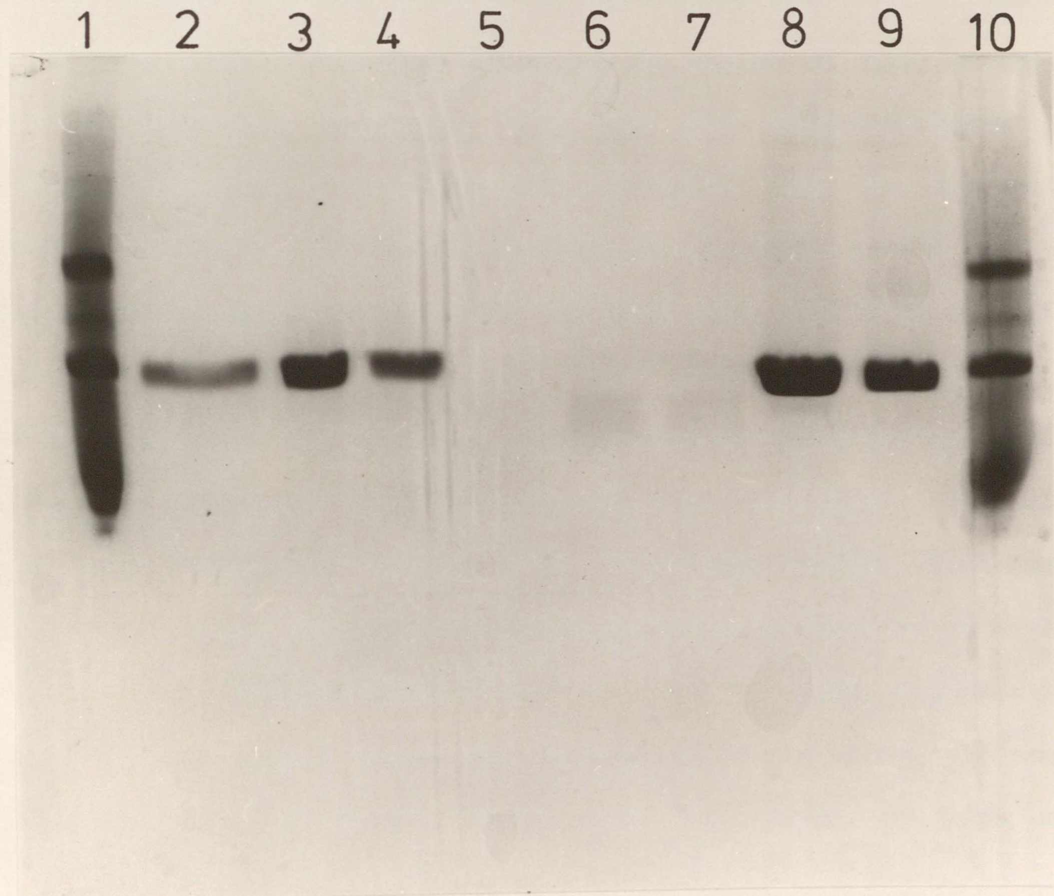
6 - not relevant

7 - not relevant

8 - fraction 10 from Fig. 5-18

9 - fraction 8 from Fig. 5-18

10- milk cell extract


1 2 3 4 5 6 7 8 9 10
−
+

1 2 3 4 5 6 7 8 9 10
−
+

5.9 Purification of the Antibacterial Compounds from the Milk Cell Extract

The milk cell extract (100 mg) was dissolved in 0.01 mol/l EDTA and chromatographed through a column of Sephadex G 75 eluted with 0.005 mol/l EDTA in 0.05 mol/l Tris buffer, pH 7.4 The elution profile showed 3 distinct areas (see Fig. 5-14), and was therefore divided up into fractions 11-15, 16-20 and 21-30. As fractions 21-30 appeared on SDS electrophoresis to be the least aggregated, they were chosen for further purification before the other fractions.

Fractions 21-30 were concentrated by rotary evaporation to a volume of 10 ml and chromatographed through a column of Sephadex G 50 eluted with distilled water. The results are shown in Fig. 5-15. Antibacterial activity was detected in fractions 11-13 and 19-24, and also in the large bed volume peak, which was attributed to the EDTA. Active fractions 19-24 (Fig. 5-15) were evaporated to 5 ml and NaCl was added to a concentration of 1.0 mol/l, to assist in the disaggregation of the compounds present. This was then chromatographed through a column of Sephadex G 75 eluted with 1.0 mol/l NaCl in 0.1 mol/l Tris buffer, pH 7.4. Two peaks were eluted (see Fig. 5-16), both of which had antibacterial activity. The two peaks were separated into fractions 11-17 and 18-22. Fractions 11-17 were concentrated by rotary evaporation to 18 ml, and 9 ml samples were re-chromatographed through a column of Sephadex G 25 eluted with distilled water. A single major peak resulted as shown in Fig. 5-17, (fractions 16-18), which was pooled from both runs. The antibacterial compound appeared to be retarded on the Sephadex G 25 column as it came out near the bed volume, and the tail of the active peak was eluted with the salts. To remove the remaining salt, fractions 16-18 were evaporated to 5 ml and

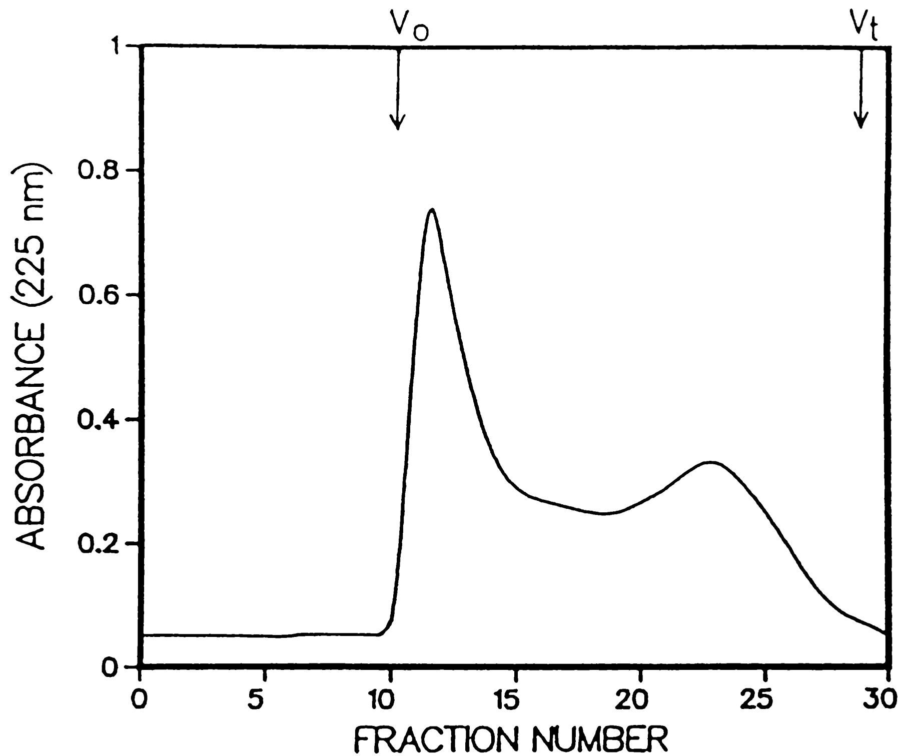


Fig. 5-14: Gel Filtration Chromatography of the Milk Cell Extract
Gel: Sephadex G 75 F
Eluent: 0.001 mol/l EDTA in 0.05 mol/l Tris buffer, pH 7.4
Sample: Milk cell extract dissolved in 5 ml of 0.01 mol/l EDTA in 0.05 mol/l Tris buffer, pH 7.4
Fraction Volume: 10 ml

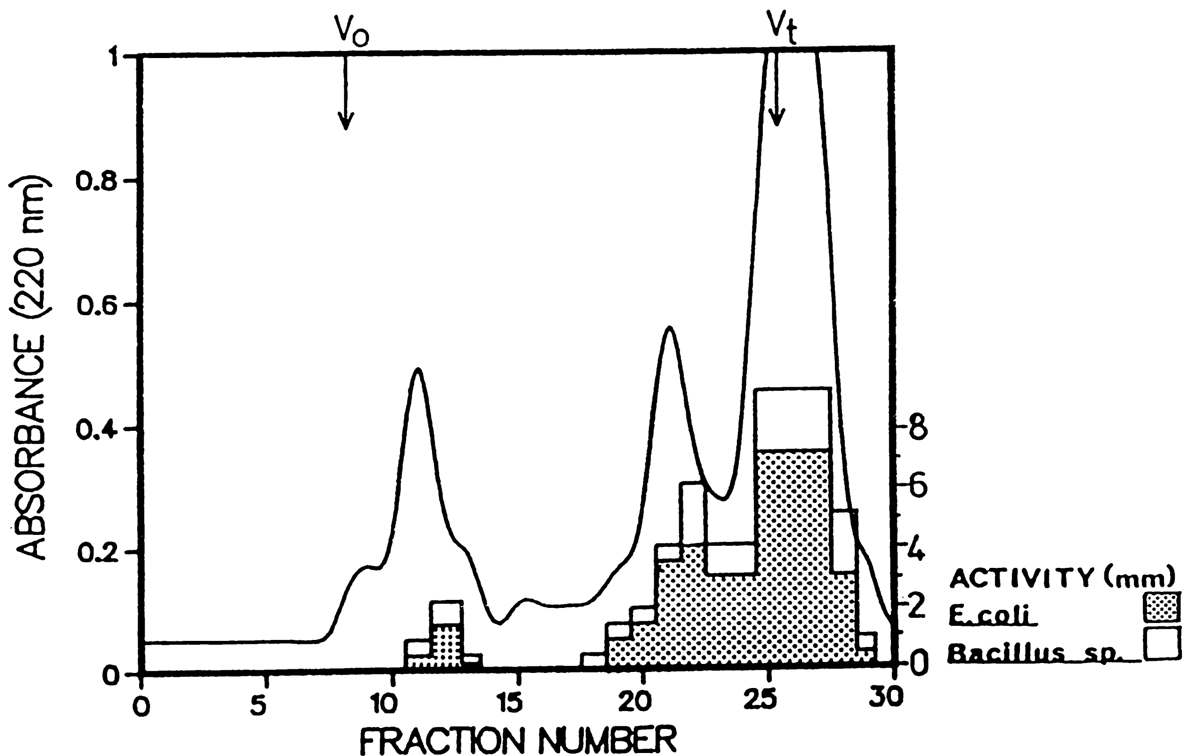


Fig. 5-15: Gel Filtration Chromatography of Fractions 21-30 from Fig. 5-14
Gel: Sephadex G 50 SF
Eluent: Distilled water
Sample: Fractions 21-30 (Fig. 5-14) evaporated to 10 ml
Fraction Volume: 10 ml

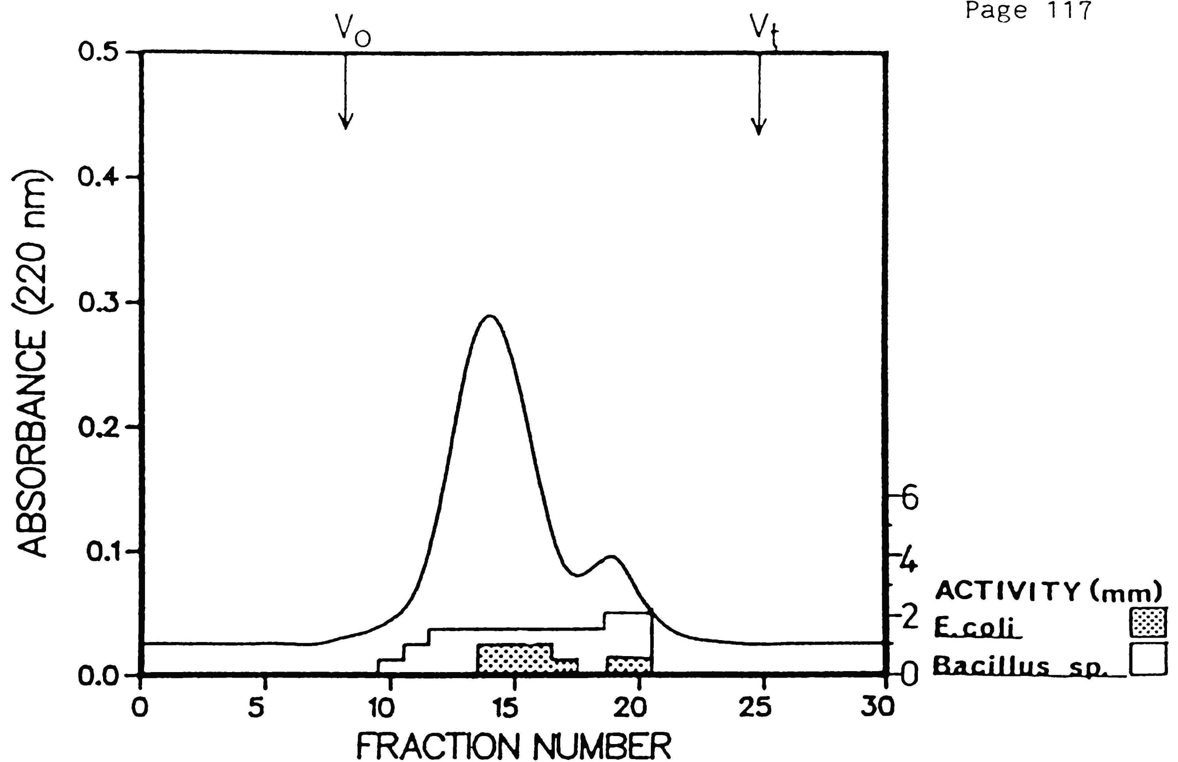


Fig. 5-16: Gel Filtration Chromatography of Fractions 19-24 from Fig. 5-15
Gel: Sephadex G 75 F
Eluent: 1.0 mol/l NaCl in 0.1 mol/l Tris buffer, pH 7.4
Sample: Fractions 19-24 (Fig. 5-15) evaporated to 5 ml and NaCl added to a concentration of 1 mol/l
Fraction Volume: 10 ml

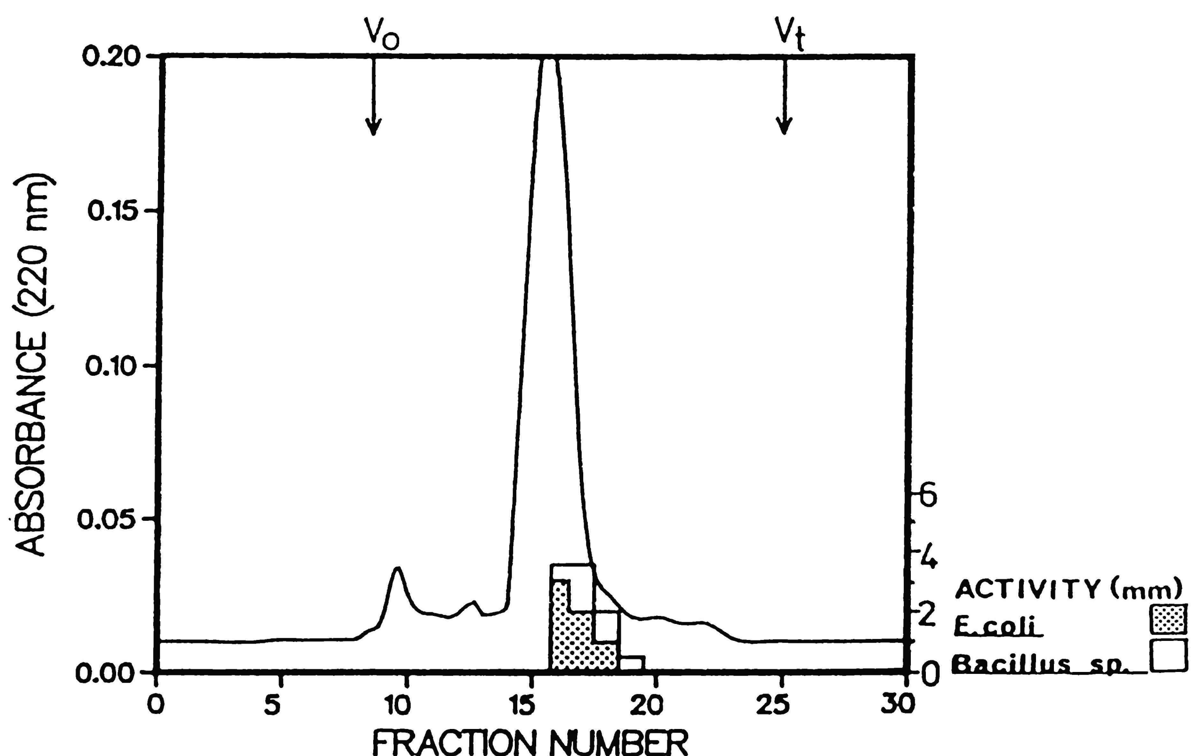


Fig. 5-17: Gel Filtration Chromatography of Fractions 11-17 from Fig. 5-16
Gel: Sephadex G 25 SF
Eluent: Distilled water
Sample: Fractions 11-17 (Fig. 5-16) evaporated to 18 ml and 9 ml samples applied to the column
Fraction Volume: 10 ml

re-chromatographed on a column of Sephadex G 15 eluted with distilled water. The results are shown in Fig. 5-18. The antibacterial compound was again retarded on the Sephadex G 15 gel, but was resolved from the salt peak. Samples (1 ml) were taken from fractions 8 and 10 and freeze-dried. They were dissolved in SDS sample buffer and separated by SDS electrophoresis. The results are shown in Plate 5-14. A single band resulted from both fractions which corresponded to the area of antibacterial activity previously found in the preparative SDS electrophoresis. Before examining the purified compound in greater detail, some of the other active peaks were investigated.

Fractions 18-22 (Fig 5-16) were pooled and evaporated to 10 ml and chromatographed through a column of Sephadex G 25 column eluted with distilled water. The results are shown in Fig. 5-19. The peak containing fractions 16-18 was the only peak with antibacterial activity. Samples (1 ml) were taken from fraction 16 - 18 and freeze-dried. These samples were dissolved in SDS sample buffer and run on SDS electrophoresis. All three samples ran as a single band with the same mobility as the active fractions from Fig. 18 (see Plate 5-14).

Fractions 11-13 (Fig. 5-15) were pooled and evaporated to 5 ml and EDTA was added to a concentration of 0.005 mol/l. These fractions were chromatographed through a column of Sephadex G 75 eluted with 0.005 mol/l EDTA in 0.05 mol/l Tris buffer, pH 7.4. The results are shown in Fig. 5-20. The elution profile showed a broad peak (fractions 17-29) which was concentrated by rotary evaporation to 10 ml and re-chromatographed on a column of Sephadex G 50 eluted with distilled water. The results are shown in Fig. 5-21. Antibacterial activity was only detected in the region of low molecular weight (fractions 18-23), presumably having been dissociated from the material of higher molecular

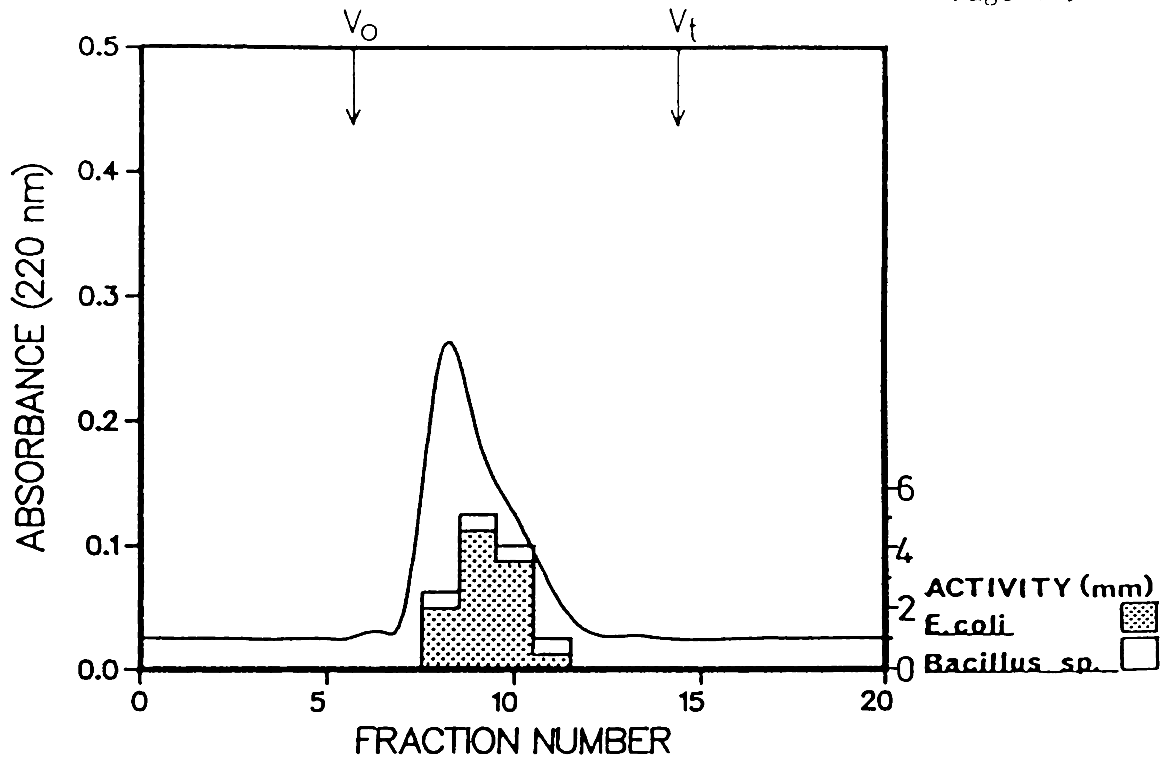


Fig. 5-18: Gel Filtration Chromatography of Fractions 16-18 from Fig. 5-17
Gel: Sephadex G 15
Eluent: Distilled water
Sample: Fractions 16-18 (Fig. 5-17) evaporated to 5ml
Fraction Volume: 10 ml

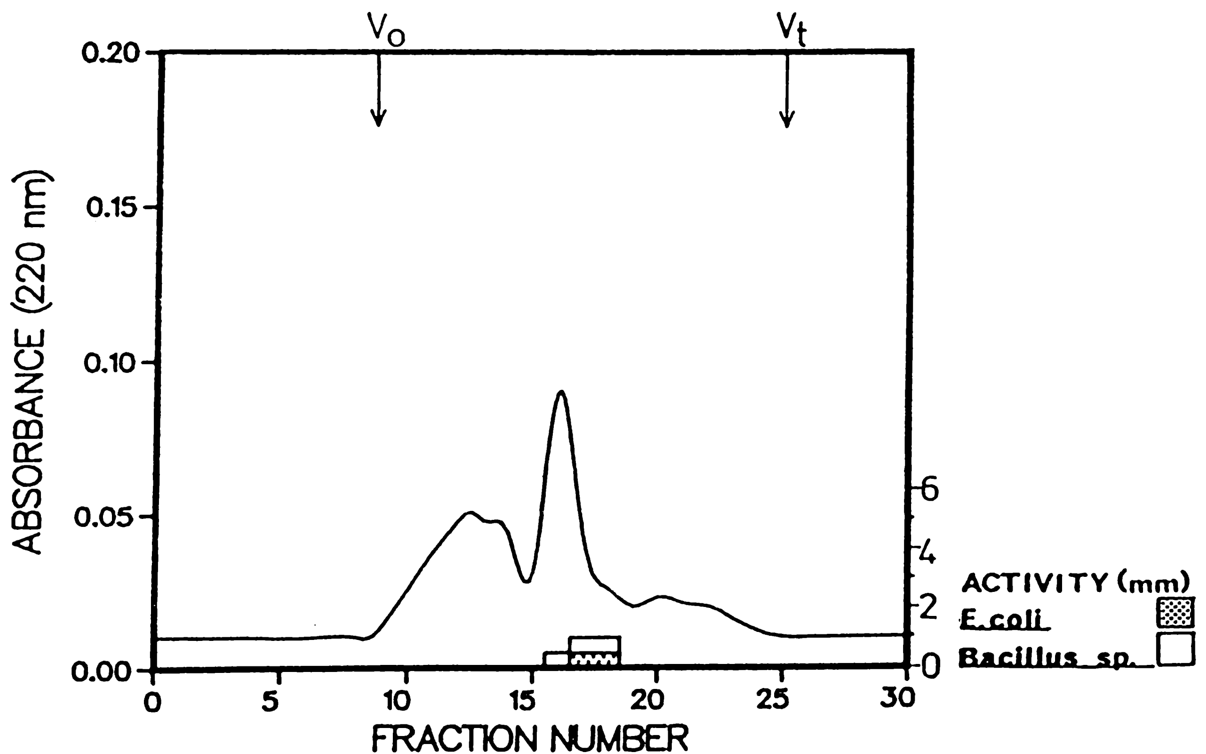


Fig. 5-19: Gel Filtration Chromatography of Fractions 18-22 from Fig. 5-16
Gel: Sephadex G 25 SF
Eluent: Distilled water
Sample: Fractions 18-22 (Fig. 5-16) evaporated to 10 ml
Fraction Volume: 10 ml

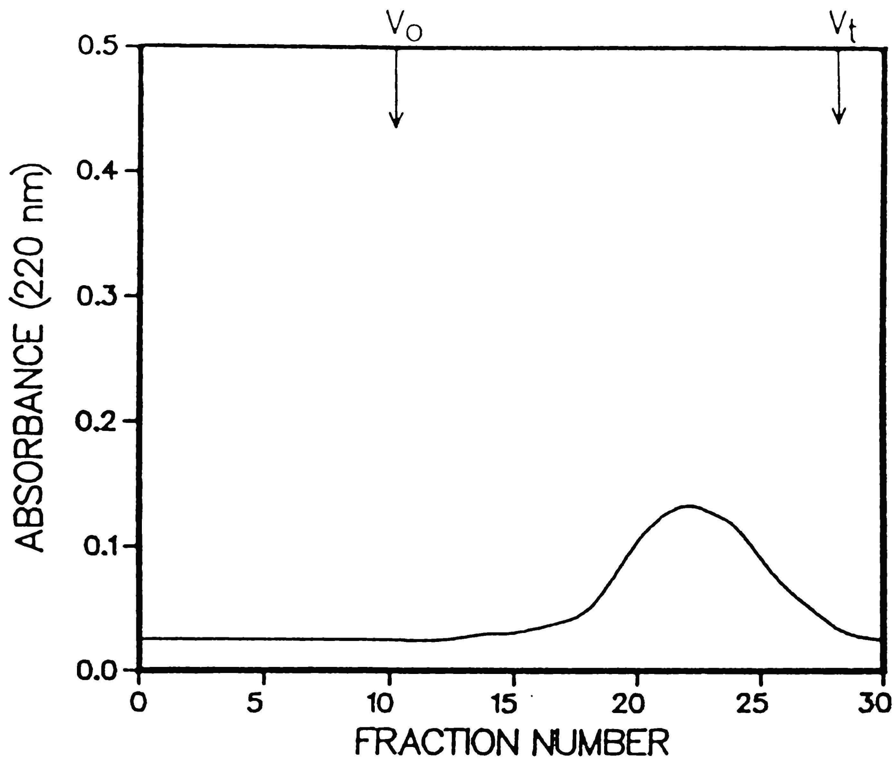


Fig. 5-20: Gel Filtration Chromatography of Fractions 11-13 from Fig. 5-15
Gel: Sephadex G 75 F
Eluent: 0.005 mol/l EDTA in 0.05 mol/l Tris buffer, pH 7.4
Sample: Fractions 11-14 (Fig. 5-15), evaporated to 5 ml, EDTA added to a concentration of 0.005 mol/l and adjusted to pH 7.4
Fraction Volume: 10 ml

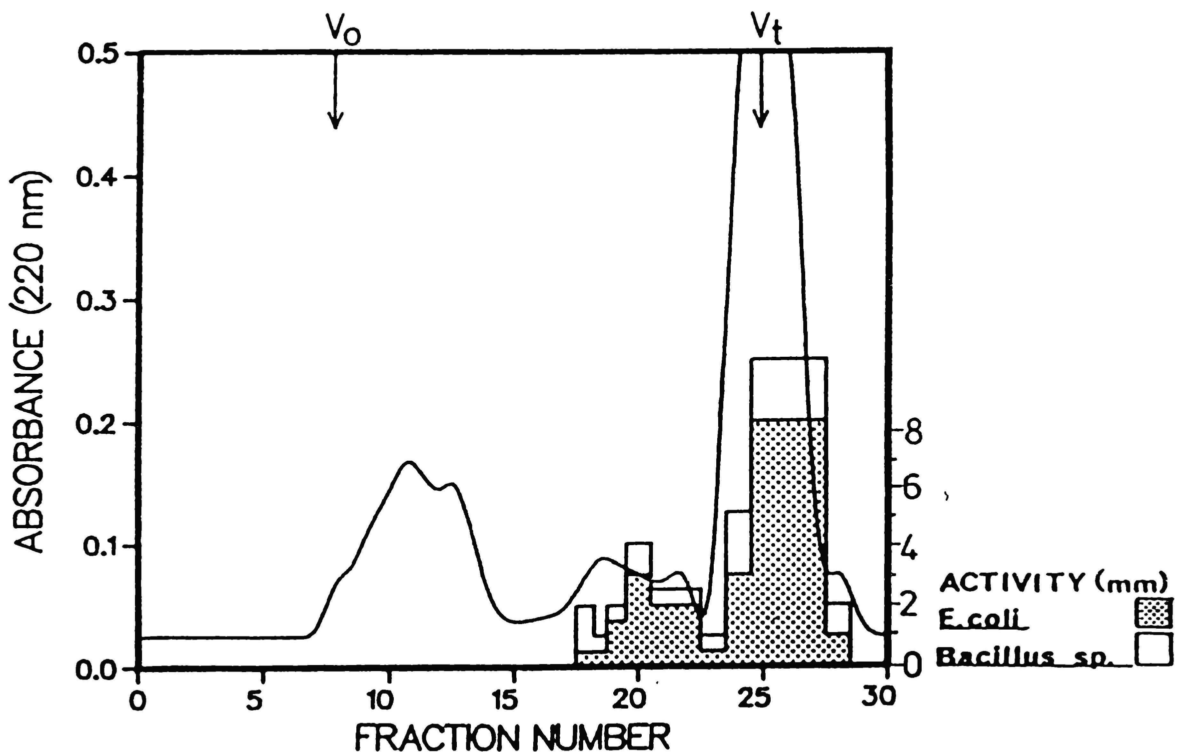


Fig. 5-21: Gel Filtration Chromatography of Fractions 17-29 from Fig. 5-20
Gel: Sephadex G 50 SF
Eluent: Distilled water
Sample: Fractions 17-29 (Fig. 5-20) evaporated to 10 ml
Fraction Volume: 10 ml

weight to which it was originally bound (see Fig. 5-15). Fractions 18-23 (Fig. 5-21) were concentrated to 5 ml by rotary evaporation, and combined with a further 5 ml of 0.1 mol/l phosphate buffer, pH 7.0. The 10 ml volume was applied to a column of SP Sephadex C 25 ion exchanger, equilibrated with 0.1 mol/l phosphate buffer, pH 7.0. The column was washed with phosphate buffer until the absorbance at 220 nm had reached baseline, and then with 0.5 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 7.0. Any remaining protein was removed with 0.1 mol/l NaOH. The results are shown in Fig. 5-22. The alkaline fractions were neutralised with HCl and all fractions were assayed for antibacterial activity. Only Fraction 4 was active. Although retarded somewhat, this fraction was eluted from the ion exchanger with the phosphate buffer and therefore must have been very weakly bound.

Fraction 4 was concentrated by rotary evaporation to 5 ml and chromatographed through a column of Sephadex G 15 eluted with distilled water (Fig. 23). A single major antibacterial peak was eluted from the void volume of the column. A 1 ml sample from the active peak (fraction 7) was freeze-dried, re-dissolved in SDS sample buffer and run on SDS electrophoresis. This sample, shown in Plate 5-15, appeared as a single band very near to the dye front suggesting the presence of a protein of low molecular weight.

The results above indicate that two distinct antibacterial proteins are present in the milk cell extract. This was substantiated when further purification was carried out of fraction 11-14 and 15-20 from the gel filtration chromatography of the milk cell extract treated with EDTA (Fig. 14). The antibacterial activity in these peaks was also found to be due to the two dissimilar antibacterial proteins.

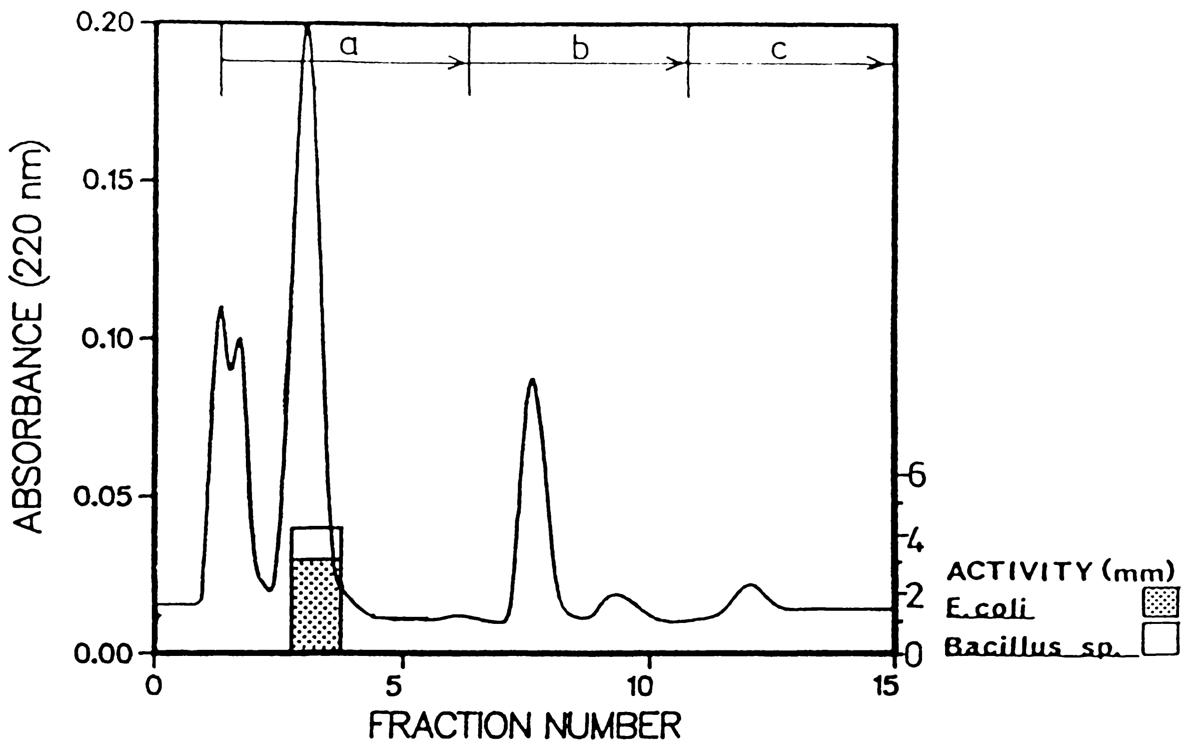


Fig. 5-22: Ion Exchange Chromatography of Fractions 18-23 from Fig. 5-21
Gel: SP Sephadex C 25
Buffer: 0.1 mol/l phosphate buffer, pH 7
Sample: Fractions 18-23 (Fig. 5-21) evaporated to 10 ml
Elution Buffers: a- 0.1 mol/l phosphate buffer, pH 7; b- 0.5 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 7; c- 0.1 mol/l NaOH
Fraction Volume: 10 ml

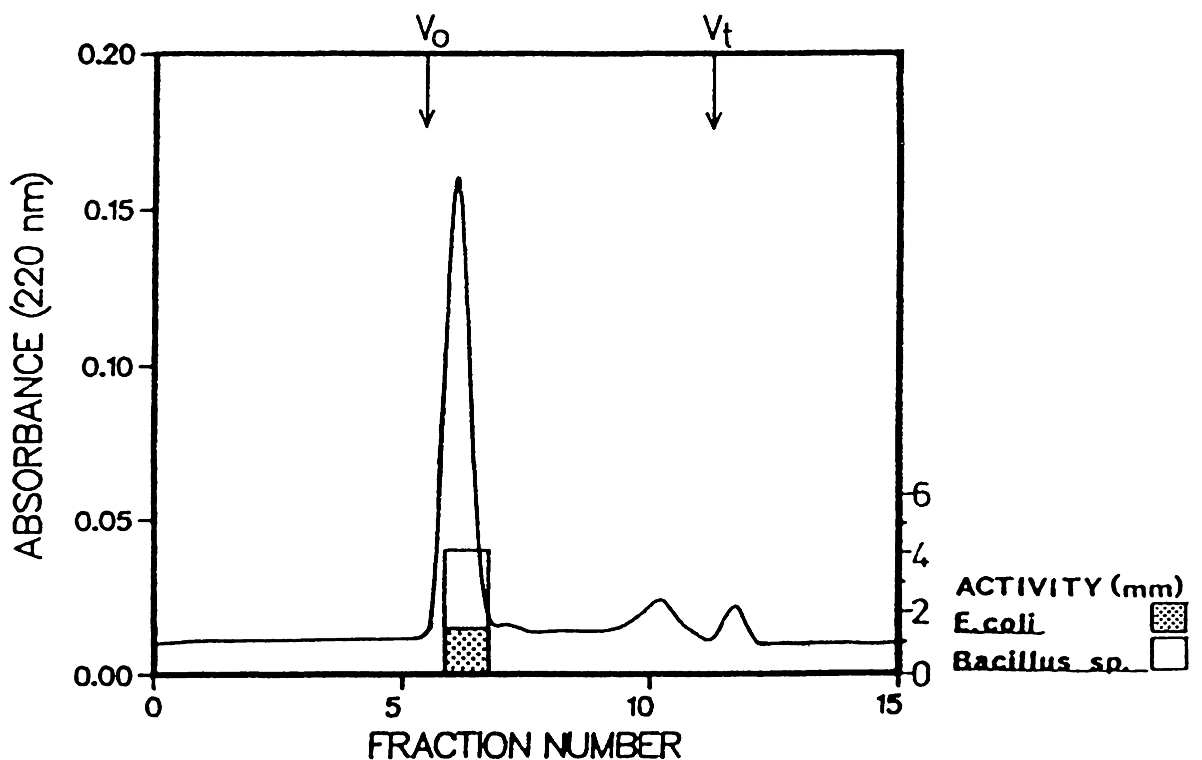


Fig. 5-23: Gel Filtration Chromatography of Fraction 4 from Fig. 5-22
Gel: Sephadex G 15
Eluent: Distilled water
Sample: Fraction 4 (Fig. 5-22) evaporated to 5 ml
Fraction Volume: 10 ml

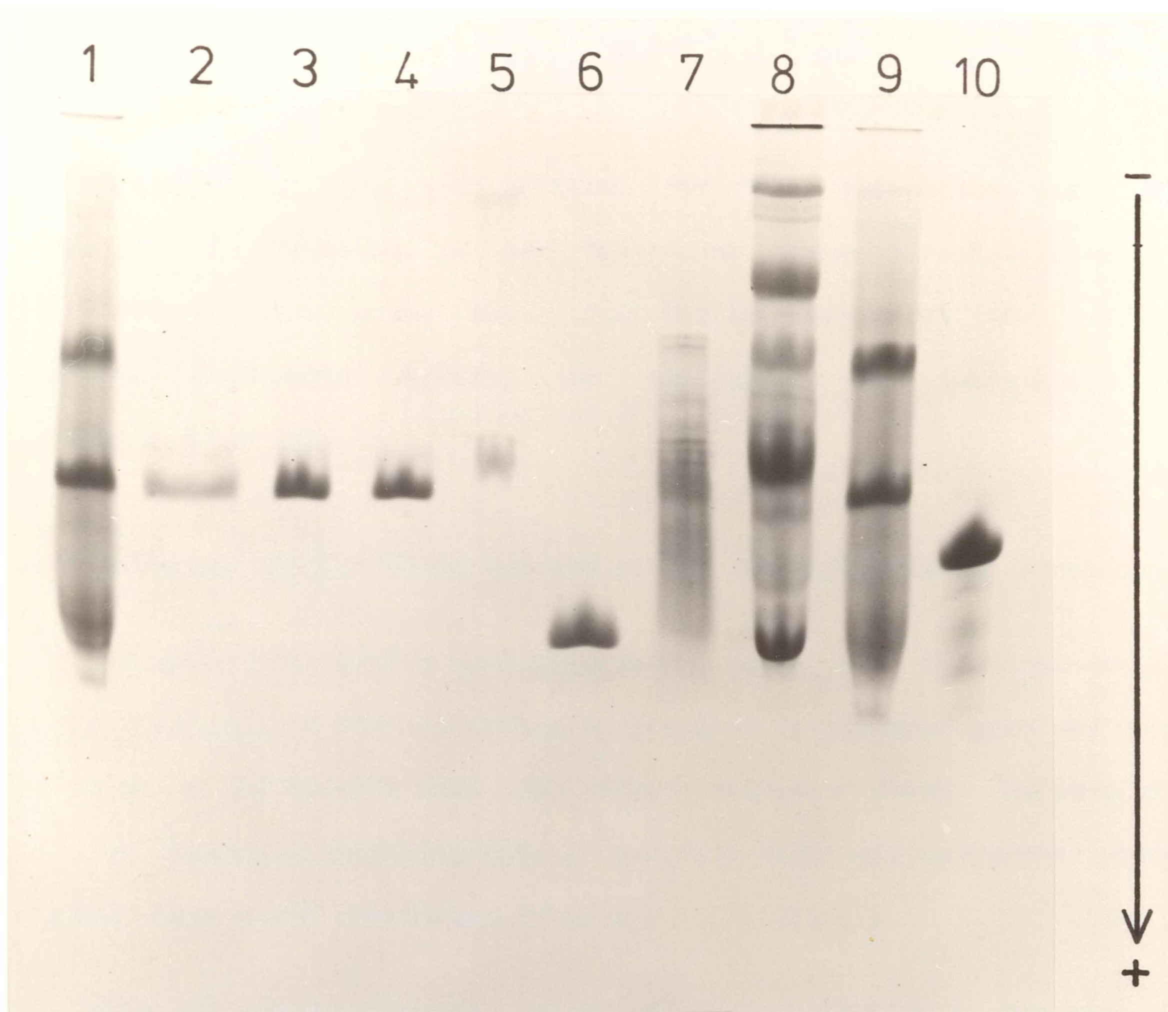


Plate 5-15: SDS Electrophoresis

1 - milk cell extract

2 - fraction 16 from Fig. 5-19

3 - fraction 17 from Fig. 5-19

4 - fraction 18 from Fig. 5-19

5 - not relevant

6 - fraction 7 from Fig. 5-23

7 - not relevant

8 - not relevant

9 - milk cell extract

10- lysozyme standard

Conclusions

The use of EDTA was critical for the separation of the antibacterial compounds by gel filtration chromatography. However, antibacterial activity was still distributed throughout the elution profile, presumably present as aggregates not susceptible to dissociation by EDTA.

5.10 Molecular Weight Determinations of the Two Antibacterial Proteins

The molecular weights were determined using SDS electrophoresis. As the behaviour of the antibacterial proteins on Sephadex gels was very dependent on the eluents used (see above), molecular weight estimations by thin layer gel chromatography or gel filtration chromatography would probably have given inaccurate values.

With SDS electrophoresis, the SDS binds to hydrophobic regions of protein molecules, destroying their tertiary structure and giving an overall negative charge to the polypeptide chains. In the presence of SDS therefore, the electrophoretic mobility of proteins in polyacrylamide gels is determined by the molecular weight of their polypeptide chains and to their extent of being "coated" with a negative charge.

Two different methods of SDS electrophoresis were used: the discontinuous SDS electrophoresis method of Laemmli (1970) run at pH 8.3, and the continuous SDS system (Sigma Chemical Company) run at pH 7.0.

Freeze-dried samples of the two antibacterial proteins were dissolved in SDS sample buffers appropriate for each of the SDS electrophoresis systems. Each of the systems were run as previously described (Chapter 2). After staining the protein bands, migration distances were measured and Rf values calculated (Plates 5-16 and 5-17). These were plotted as the logarithm of molecular weight versus R_f (Fig. 5-24 and 5-25). The molecular weights for the antibacterial proteins were calculated as:-

21 000 and 11 000 - on Sigma SDS system

17 000 and 11 000 - on Laemmli SDS system

The molecular weight variation of the larger protein (Higher Molecular Weight protein; HMW) might be due to the degree of binding of the SDS. At pH 7.0 the protein would be more cationic, which would result in a lower net negative charge on the molecule, resulting in a slower migration. With the Lower Molecular Weight (LMW) protein the molecular weight value was consistent with both systems.

5.11 Isoelectric Point Determination of the HMW and LMW Proteins

Isoelectric focusing was carried out using LKB Ampholine PAGplates with a pH 3.5 to 9.5 gradient, and Servalyte-Precotes with a pH 3 to 10 gradient.

Samples were desalted by column chromatography and freeze-dried. They were re-dissolved in distilled water and applied to the surface of the Ampholine PAGplate using the paper strips supplied. Constant power (5 W) was applied for 2 hours until the standards were focused and the ampholine bands across the gel were clearly visible. The resulting stained gel (see Plate 5-18) showed the HMW protein as a band at the cathode, which indicated that it had a pI greater than 9.5. The LMW

Plate 5-16: SDS Electrophoresis

1 - lysozyme, trypsinogen, carbonic anhydrase and ovalbumin standards

2 - not relevant

3 - fraction 7 from Fig. 5-23

4 - fraction 7 from Fig. 5-23

5 - fraction 17 from Fig. 5-19

6 - fraction 17 from Fig. 5-19

7 - not relevant

8 - not relevant

9 - milk cell extract

10- BDH molecular weight standards

Plate 5-17: SDS Electrophoresis

1 - lysozyme, trypsinogen, carbonic anhydrase and ovalbumin standards

2 - cytochrome c, myoglobin, ribonuclease and α-chymotrypsinogen standards

3 - milk cell extract

4 - fraction 17 from Fig. 5-19

5 - fraction 17 from Fig. 5-19

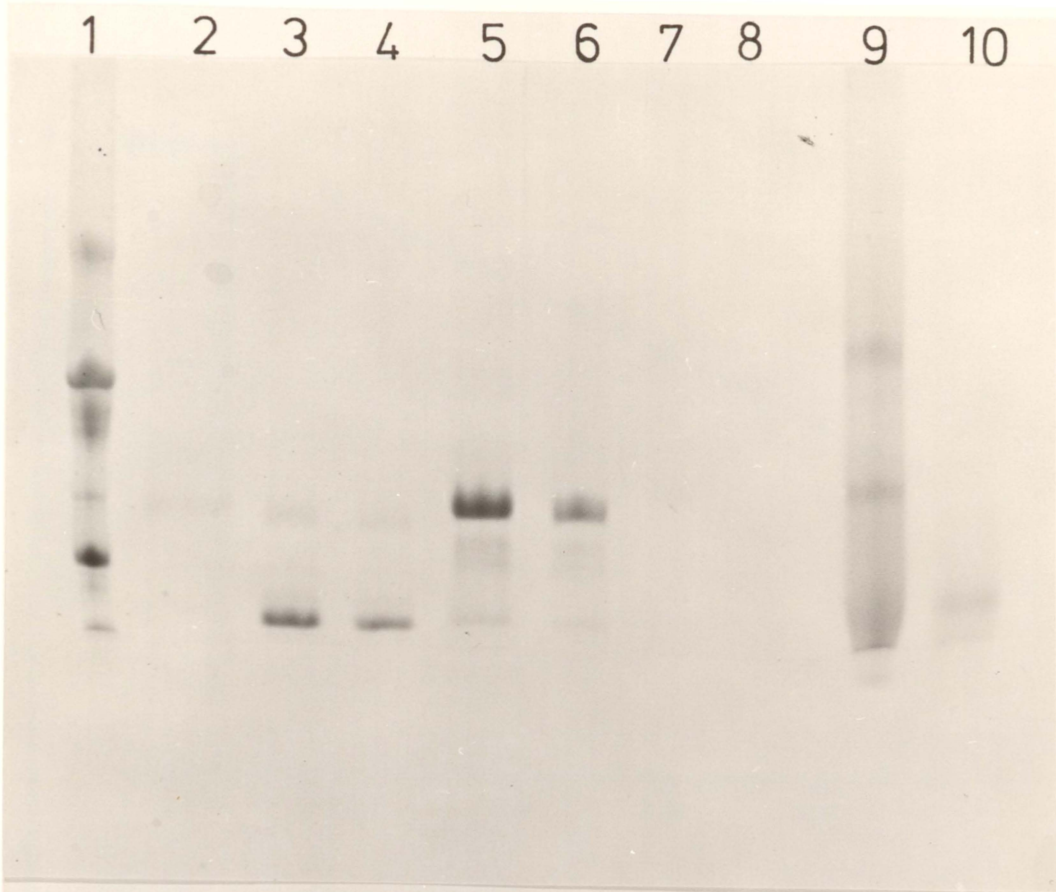
6 - fraction 7 from Fig. 5-23

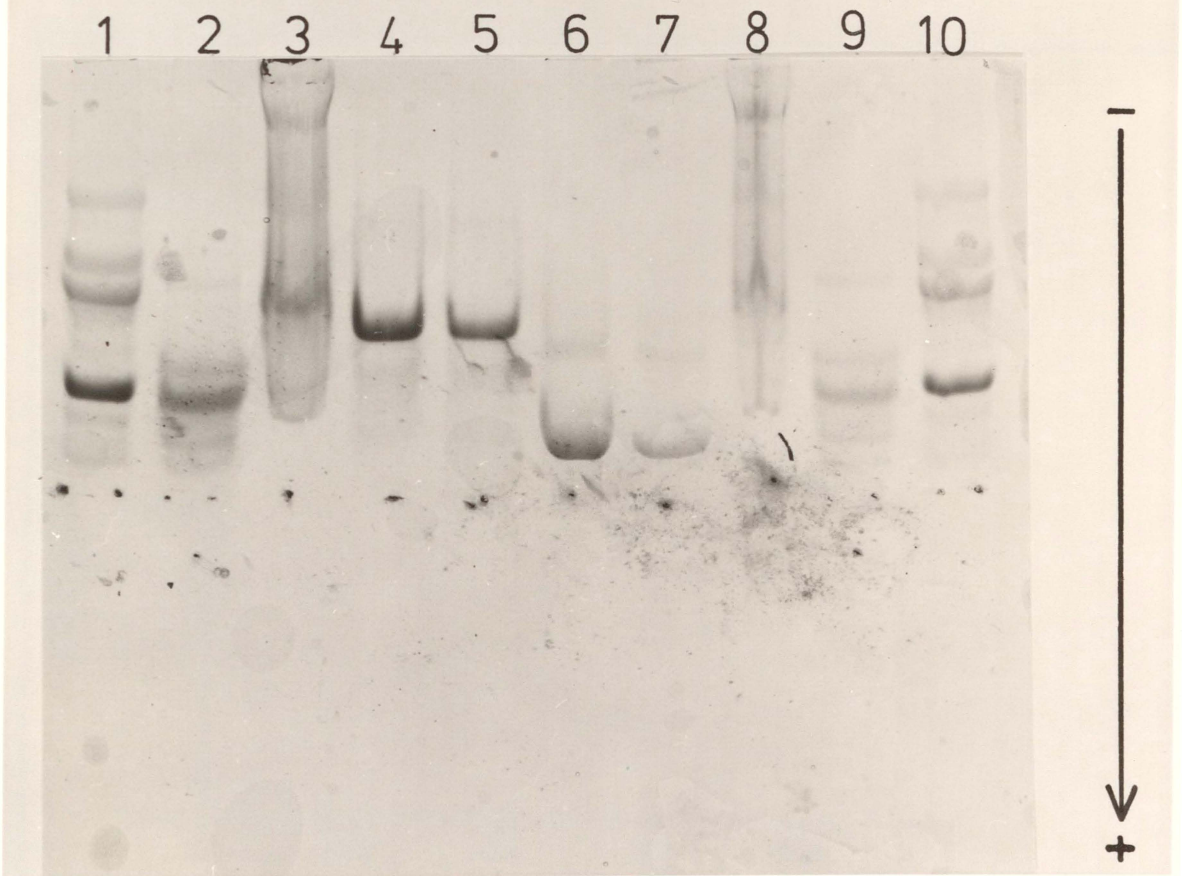
7 - fraction 7 from Fig. 5-23

8 - as in 3

9 - as in 2

10- as in 1


1 2 3 4 5 6 7 8 9 10
−
+


1 2 3 4 5 6 7 8 9 10
−
+

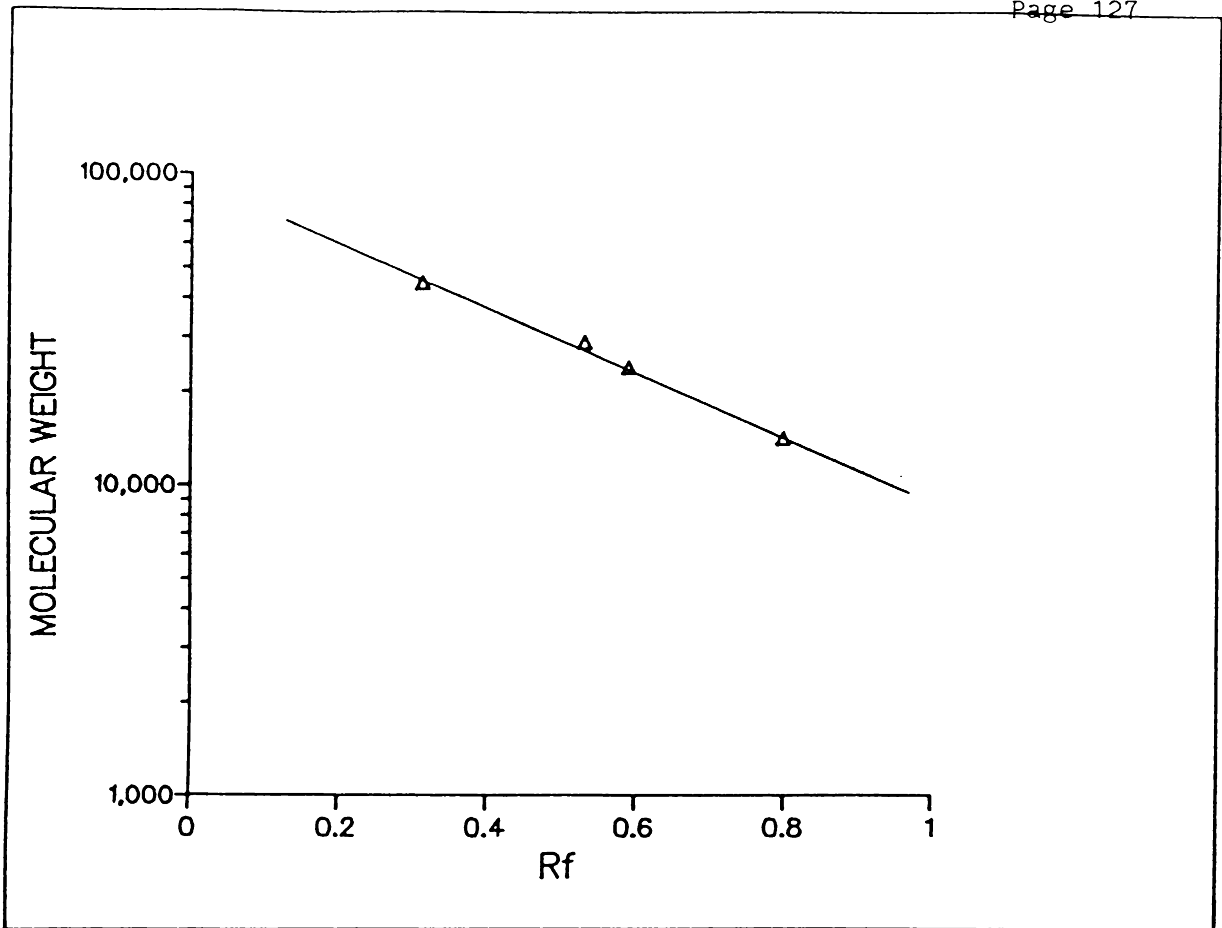


Fig. 5-24: Calibration Curve of Standards Run on SDS Electrophoresis (Laemmli system, Plate 5-16)

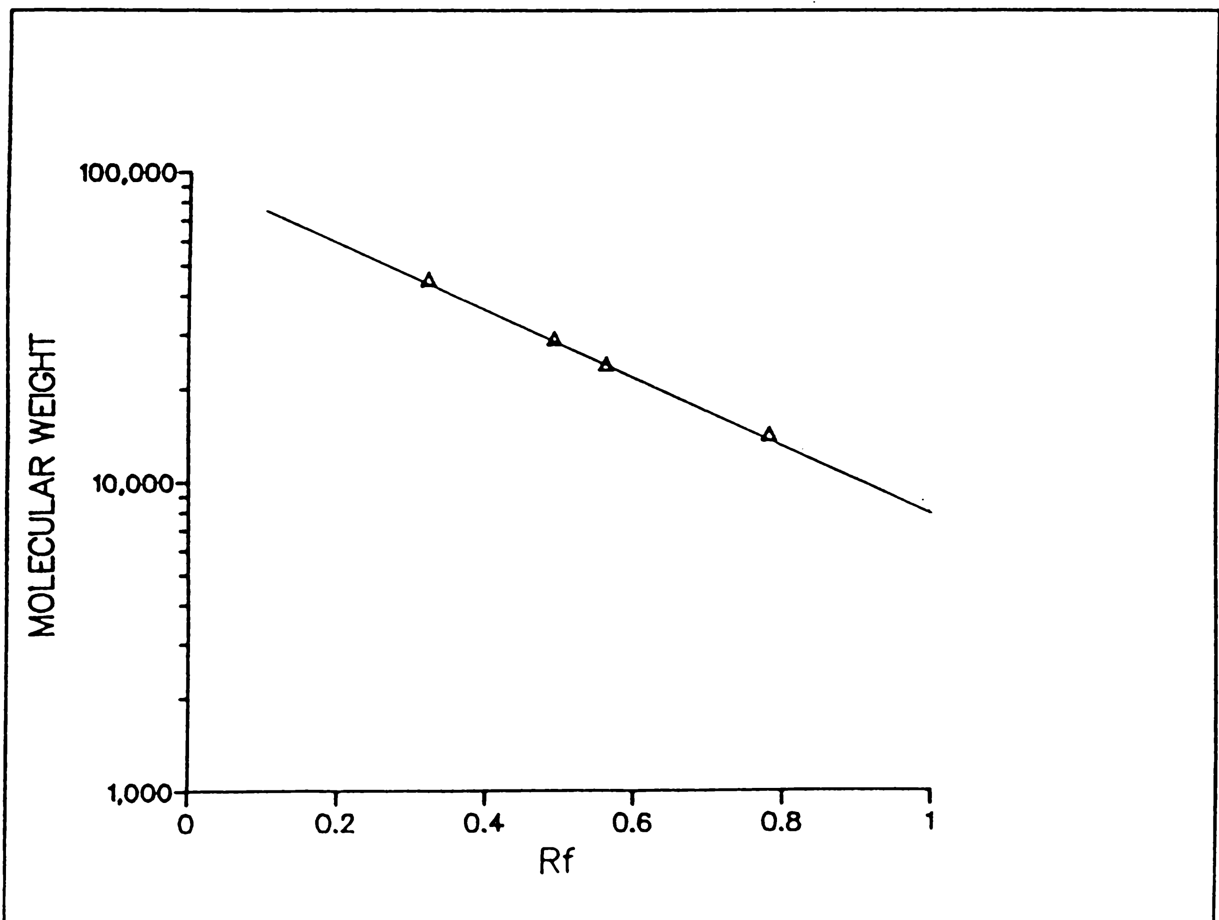


Fig. 5-25: Calibration Curve of Standards run on SDS Electrophoresis (Sigma system, Plate 5-17)

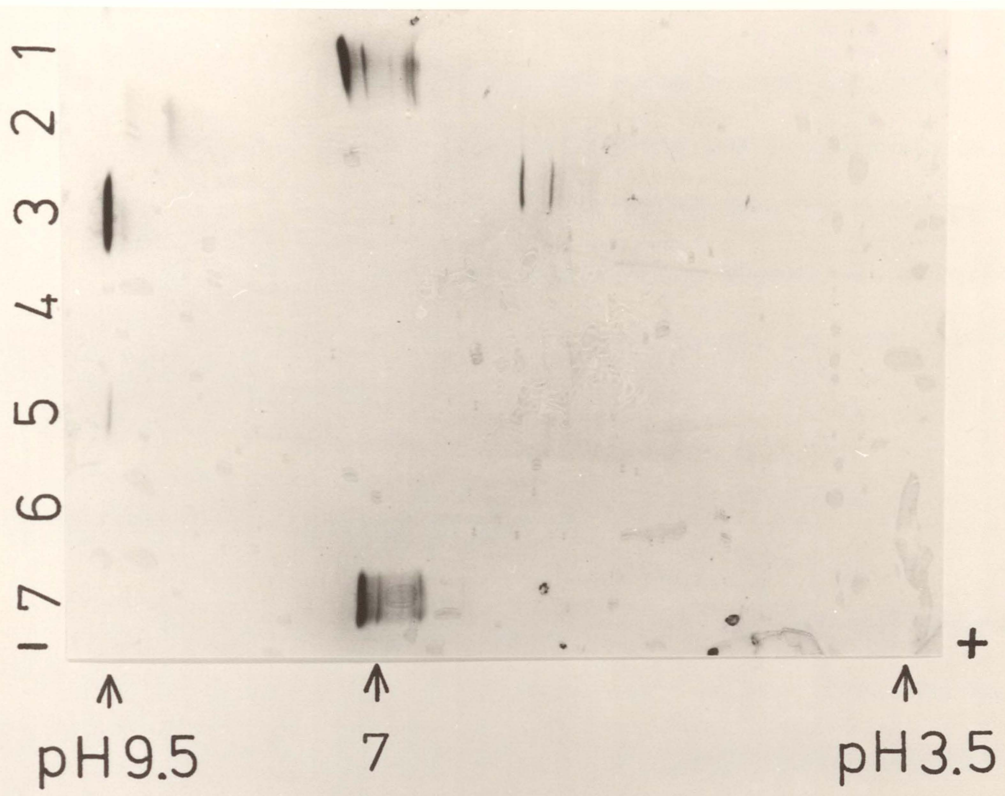


Plate 5-18: Isoelectric Focusing

1 - myoglobin standard

2 - LMW protein

3 - HMW protein

4 -

5 - cytochrome c standard

6 -

7 - myoglobin standard

protein gave a series of bands between pH 6 and 7, and two other bands near the cathode. It was concluded that the LMW protein was probably still heterogeneous. However the apparent heterogeneity could be due to artifacts generated by interaction with carrier ampholytes or electrode modification from a sample focusing too close to the electrodes (Righetti and Drysdale, 1976).

5.12 Determination of Lysozyme Activity

Hibbitt and coworkers (1971) reported that lysozyme was absent from the proteins extracted from the cells in cows' milk. This was based on the failure of the milk cell extract to lyse Micrococcus lysodeikticus, and the absence of a band migrating towards the cathode at pH 9.0 during electrophoresis (lysozyme has a pI 10.5-11.0). However Eschenbruch (1980) found that lysozyme was initially inactive in the antibacterial calf thymus preparation, but activated by removal of an uncharacterised inhibitor during purification.

The two antibacterial proteins purified from the milk cell extract were assayed for lytic activity by the lyso-plate method of Osserman and Lawler (1966). This assay is based on the lysis of pre-grown Micrococcus lysodeikticus cells in nutrient agar plates. A similar, more sensitive lyso-plate assay (Gosnell et al., 1975) was also used, which measured the lytic activity on lyophilised Micrococcus lysodeikticus cells.

Active samples from the final purification steps of the Higher Molecular Weight (Fig. 5-18) and Lower Molecular Weight (Fig. 5-23) proteins were placed in sample wells cut from the plates. After incubating for 12 hours or overnight the zones of clearing were measured

(Table 5-1).

From these results it appears that the HMW protein has some lytic activity although, without further characterisation, it cannot be called a lysozyme. The HMW protein also differs in molecular weight from the lysozyme-like protein isolated from bovine seminal plasma.

5.13 Interaction between the HMW and LMW Proteins in their Antibacterial Activities

The antibacterial activities of the HMW and LMW proteins were measured singly and in combination to determine whether any activation or inhibition occurred ofter mixture.

Active samples of both proteins were combined as shown in Table 5-2. It appears from these results that the HMW and LMW proteins have no interaction which alters their degree of activity.

5.14 Conclusions

The milk cell extract of Hibbitt and coworkers (1971) owes its activity to two antibacterial substances; the Higher Molecular Weight (HMW) protein and the Lower Molecular Weight (LMW) protein. These antibacterial proteins are released from an aggregated state in the milk cell extract by gel filtration chromatography in the presence of EDTA. The exact action of the EDTA on the milk cell extract is unclear. The HMW protein has lytic activity which is not apparent in the original extract (possibly because of inhibition by other substances present).

	Clearing (mm)	
	Pregrown M. lysodeikticus	Lyophilised M. lysodeikticus
HMW Protein	2.0	3.5
LMW Protein	0	0
Lysozyme	3.0	5.0

Table 5-1: Lytic activity of the HMW and LMW proteins measured as the clearing (mm) on pre-grown and lyophilised *M. lysodeikticus* seeded agarose plates

	Clearing (mm)	
	Bacillus Sp.	E. coli
HMW 1:1 water	1.5	1.5
LMW 1:1 water	3.0	2.0
HMW 1:1 LMW	2.5	1.0

Table 5-2: Antibacterial activity of the HMW and LMW proteins on bacteria seeded agarose plates

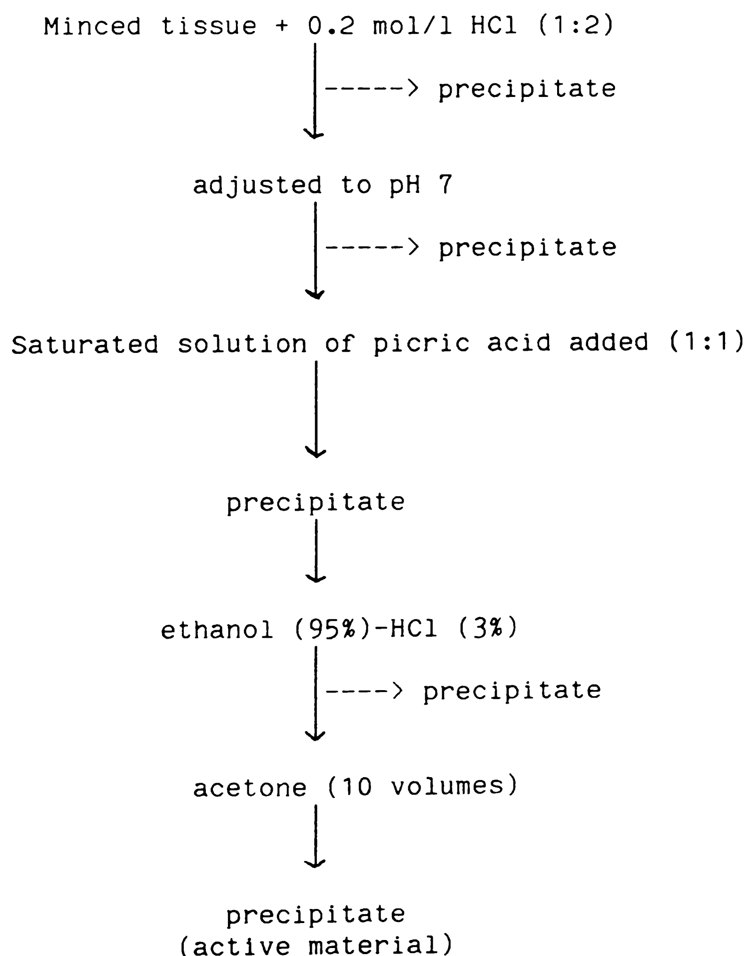
The HMW protein has a molecular weight of between 17 000 and 21 000 and an isoelectric point greater than pH 9.5. It is similar in many respects to the lysozyme-like protein purified from bovine seminal plasma. The LMW protein has a molecular weight of 11 000 but on isoelectric focusing gives several bands. There is apparently no interaction between the two proteins as far as enhancing or inhibiting their overall antibacterial activities.

CHAPTER SIX

INVESTIGATION OF THE SHEEP THYMUS PEPTIDE

6.1 Introduction

In 1954, Dubos and Hirsch described a method for the isolation of antibacterial material from a number of tissues. Active fractions could be derived from calf thymus, spleen and pancreas, as well as from sheep thymus. Antibacterial activity was determined with mycobacterium as a test organism. No lytic activity was detected in the preparation so lysozyme was presumed not to be responsible for any of the antibacterial activity. The procedure used for the isolation of the activity is outlined below.



6.2 Properties of the Antibacterial Preparation described by Hirsch and Dubos (1954)

The calf thymus preparation, a white powder, is readily soluble in water, reasonably soluble in methanol and ethanol, and insoluble in acetone and diethylether. In a dry state the preparation is very stable, even at room temperature not losing activity for at least one year. In solution, the activity slowly decreases even when stored below 10°C. However, autoclaving with the bacterial growth medium - a liquid medium containing inorganic salts and asparagine - did not alter the inhibitory properties of the preparation. The isoelectric point of the calf thymus preparation was estimated by precipitation experiments, and it was found that the solubility was minimal between pH 10 and 11.

The molecular weight of the antibacterial compound was not determined. However, it was observed that most of the activity was lost during dialysis. Furthermore, the preparation formed a stable picrate derivative, which, according to Hirsch and Dubos (1954), is a characteristic of small proteins.

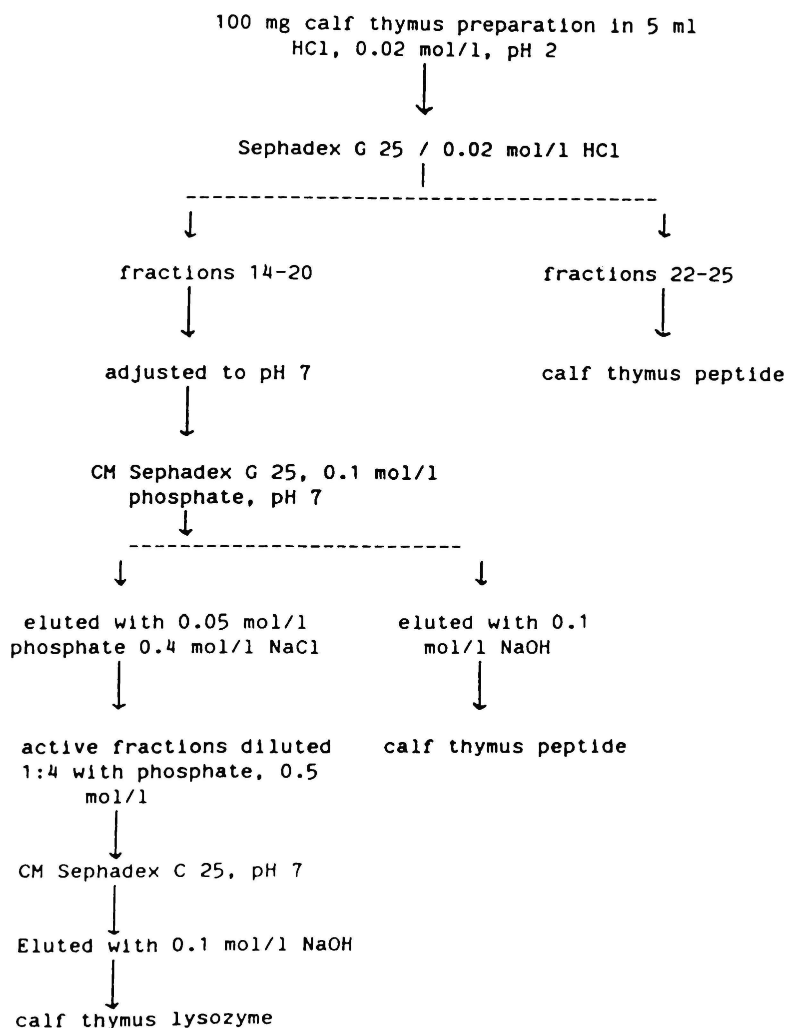
From experiments with proteolytic enzymes it was concluded that the calf thymus preparation consists of protein. This was further supported by the ultraviolet absorption spectrum in which there was maximum absorbance at 220 nm and 277-280 nm, indicating a protein-like compound. It was also shown that carbohydrate and phosphorus were absent, thus eliminating the possibility of the calf thymus preparation being a glycoprotein or polynucleotide.

The preparation was also shown to be heterogeneous. Results from amino acid analysis did not show whole number relationships between the molar ratios of many amino acids. Heterogeneity was also confirmed by an ion exchange chromatography on a cation exchange resin.

The calf thymus preparation also exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria.

6.3 Characterisation of the Antibacterial Activity from Bovine Thymus

In 1980, Eschenbruch found that the antibacterial activity of the calf thymus preparation of Dubos and Hirsch (1954) was due to two substances, lysozyme and a cationic peptide. Eschenbruch also found that the sheep thymus preparation was identical to the calf thymus preparation. The method developed by Eschenbruch (1980) to isolate lysozyme and the cationic peptide from these preparations is outlined below.



Eschenbruch (1980) also compared the cationic peptides from calf thymus, beef spleen, seminal plasma and rumen and found that they behaved in an identical manner on cationic electrophoresis. The rumen "peptide" was subsequently identified as spermine (Briggs, 1983), and this therefore brought into question the identity of the other peptides. It was also noticed that on cationic electrophoresis small cationic peptides and spermine have similar migration rates and therefore this is not a good criterion to use for comparison. The purpose of this investigation was to isolate and identify the calf thymus peptide previously isolated by Eschenbruch (1980).

6.4 Isolation and Identification of the Sheep Thymus Peptide

The sheep thymus preparation was prepared according to the method of Dubos and Hirsch (1954). The preparation displayed strong antibacterial activity but exhibited no lytic activity, as described by Dubos and Hirsch (1954) and Eschenbruch (1980). The sheep thymus preparation was then run through the purification method of Eschenbruch to obtain the thymus peptide and lysozyme.

A 100 mg sample of the sheep thymus preparation was dissolved in 5 ml of 0.02 mol/l HCl, pH 1.7, and chromatographed through a column of Sephadex G 25 eluted with 0.02 mol/l HCl (Fig. 6-1). Fractions were assayed for antibacterial activity by taking 100 µl samples of the fractions and adding this to 100 µl of 0.1 mol/l phosphate buffer pH 7. Samples (50 µl) of the thus neutralised solutions were then placed in wells on seeded agarose plates which were then incubated at 37°C for 12 hours, and the zones of inhibition measured. Two areas of activity were found, fractions 14-22 and fractions 25-26. According to Eschenbruch (1980) the active fractions 14-22 contain lysozyme and the thymus

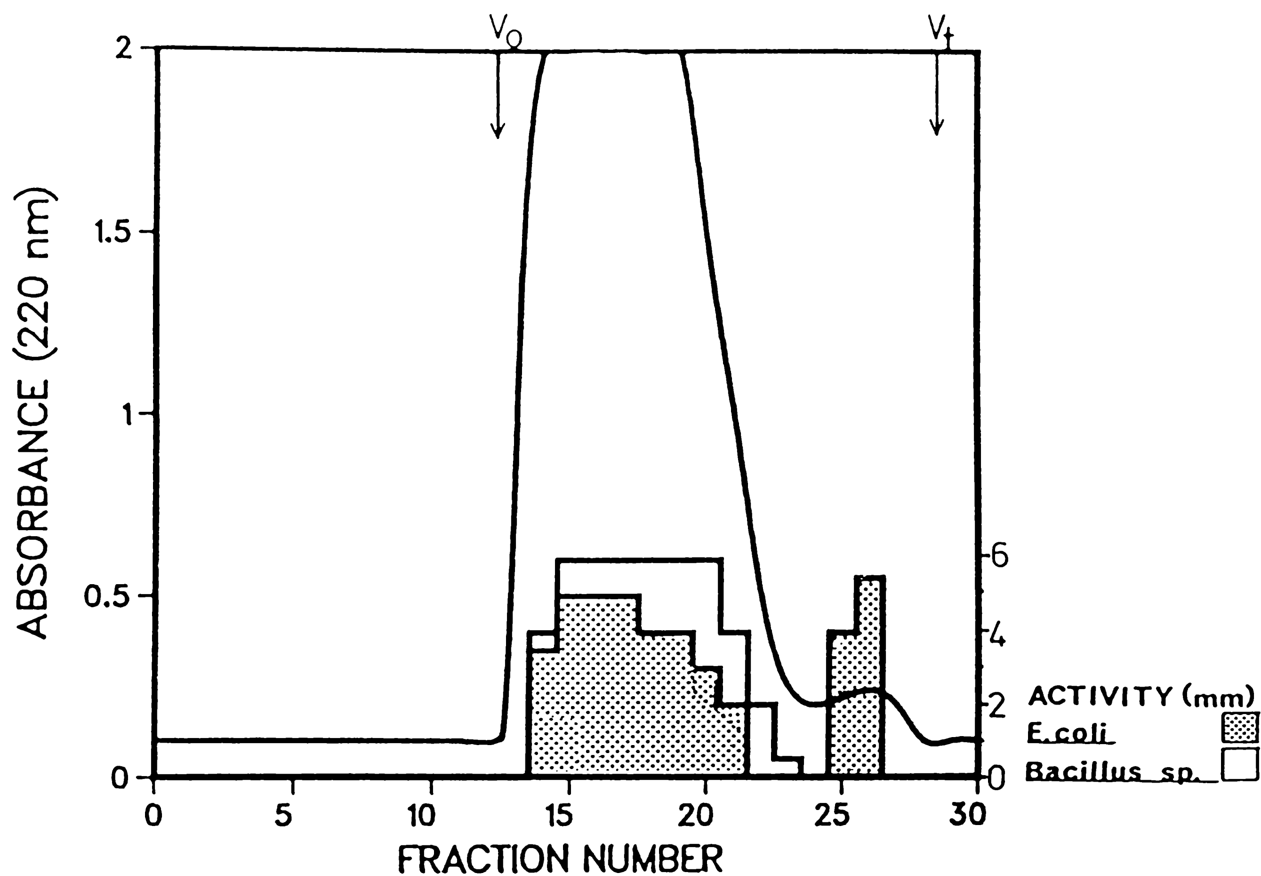


Fig. 6-1: Gel Filtration Chromatography of the Calf Thymus Preparation
Gel: Sephadex G 25 SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: 100 mg of thymus preparation dissolved in 5 ml of 0.02 mol/l HCl
Fraction Volume: 10 ml

peptide while the fractions 25-26 contain only the thymus peptide.

6.4.1 Isolation of Spermine

As fractions 25-26 contain only the thymus peptide, they were examined first for the presence of spermine by using thin layer chromatography. Fractions 25-26 were pooled and a sample of 1 ml was freeze-dried. This freeze-dried sample (approximately 1 mg) was re-dissolved in 15 µl of distilled water and 2 µl of this was run on thin layer chromatography (Fig. 6-2). The developed plate revealed the presence of both spermine and spermidine. However there was also a small amount of streaking present which was probably due to peptides co-eluting with the polyamines.

To find if the peptides were contributing to the antibacterial activity, further separation was carried out. The pooled fractions 25-26 were neutralised by the addition of 10 ml of 0.1 mol/l phosphate buffer and sufficient 1.0 mol/l HCl to adjust the pH to 7. This was then run onto a column of CM Sephadex C 25 equilibrated with 0.05 mol/l phosphate buffer pH 7. The column was eluted with a salt gradient of 0 to 1.0 mol/l NaCl in phosphate buffer pH 7, followed by a final wash with 0.1 mol/l NaOH (Fig. 6-3). After neutralising the alkaline fractions, all fractions were assayed for antibacterial activity. No activity was detected initially. However after diluting the salt-containing fractions with an equal volume of water an area of activity was detected. This was at the tail end of the eluted protein, the rest of which which did not have any detectable activity. The active fractions shown in Fig. 6-3 were evaporated to 5 ml and chromatographed through a column of Sephadex G 25 SF eluted with 0.02 mol/l HCl, pH 1.7 (Fig. 6-4). This final gel filtration run

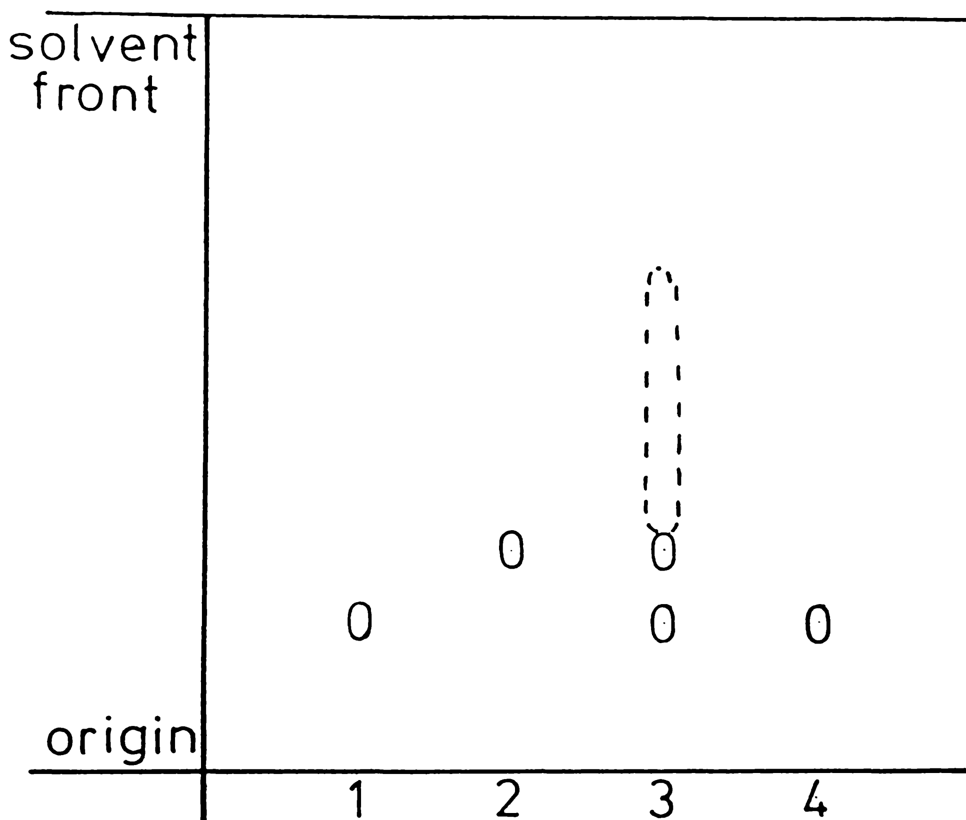


Fig. 6-2: Thin-layer Chromatography
1 - spermine standard
2 - spermidine standard
3 - fractions 25-26 from Fig. 6-1
4 - spermine standard

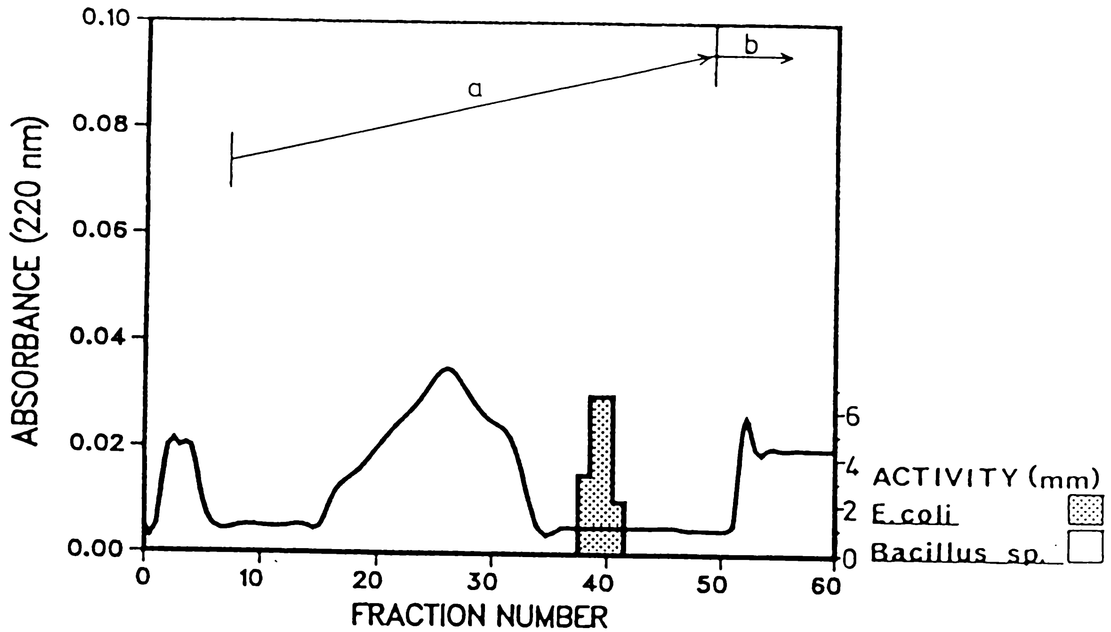


Fig. 6-3: Ion Exchange Chromatography of Fraction 25-26 from Fig. 6-1
Gel: CM Sephadex C 25
Buffer: 0.1 mol/l phosphate, pH 7
Sample: Fractions 25-26 (Fig. 6-1) adjusted to pH 7
Elution Buffers: a- salt gradient from 0 to 1 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 7; b- 0.1 mol/l NaOH
Fraction Volume: 10 ml

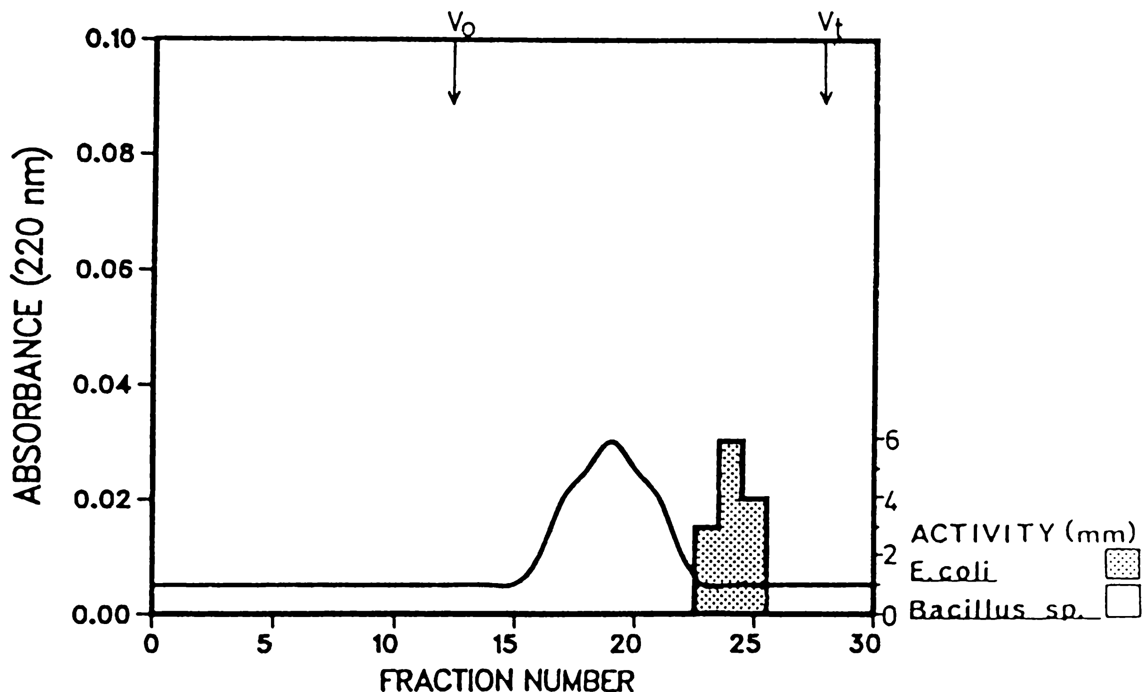


Fig. 6-4: Gel Filtration Chromatography of the Active Fractions from Fig. 6-3
Gel: Sephadex G 25 SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Active fractions (Fig. 6-3) evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

separated any remaining inactive protein and salt present from the ion exchange chromatography. There was no absorption at 220 nm in the peak of activity, this being indicative of spermine and spermidine.

Fractions comprising the peak of activity shown in Fig. 6-4 were pooled and freeze-dried. A sample (1 mg) of the freeze-dried material was dissolved in 50 µl of distilled water and a 1 µl sample was run on thin layer chromatography. After staining with ninhydrin and incubating at 110°C for 10 minutes, the material from Fig. 6-4 gave spots corresponding to spermine and spermidine with no streaking present.

6.4.2 Investigation of the Component of Higher Molecular Weight

Having established the presence of free spermine and spermidine in the thymus preparation, the investigation was now centered around whether the peptide derived from the fractions of higher molecular weight (fractions 14-22 in Fig. 6-1) was in fact these polyamines as protein or peptide complexes. The presence of an antibacterial spermine-protein complex would lend support to the argument that such complexes are a relevant factor in non-specific immunity.

The method of isolating lysozyme and the calf thymus peptide established by Eschenbruch (1980) was followed (Section 6.3). The pooled fractions 14-22 (Fig. 6-1) which contained no free spermine were adjusted to pH 7 and run onto a CM Sephadex C 25 column equilibrated with 0.1 mol/l phosphate buffer pH 7. The column was eluted with 0.05 mol/l phosphate buffer pH 7 followed by 0.4 mol/l NaCl in the same buffer. The column was finally eluted with 0.1 mol/l NaOH to remove any remaining material (Fig. 6-5). Antibacterial activity was detected in the peak removed by the wash with 0.4 mol/l NaCl and in the peak resulting from the wash with NaOH. A large degree of tailing occurred

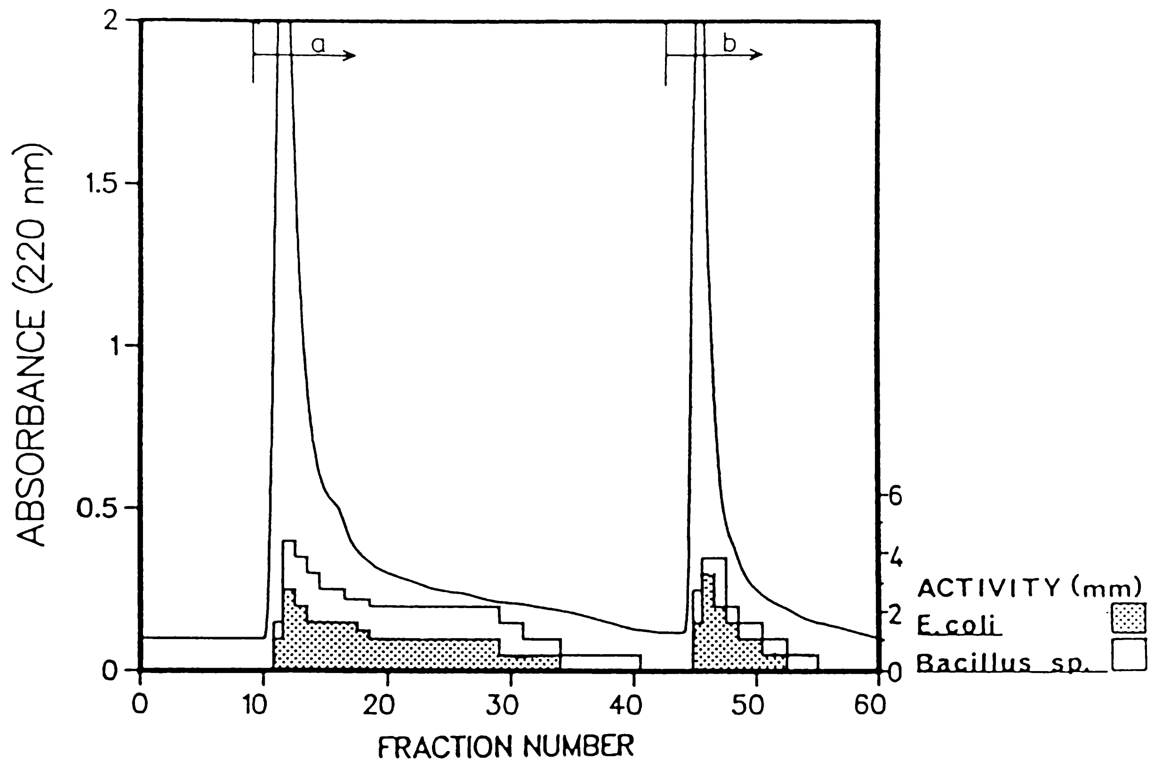


Fig. 6-5: Ion Exchange Chromatography of Fractions 14-22 from Fig. 6-1
Gel: CM Sephadex C 25
Buffer: 0.1 mol/l phosphate, pH 7
Sample: Fractions 14-22 (Fig. 6-1) adjusted to pH 7
Elution Buffers: a- 0.4 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 7; b- 0.1 mol/l NaOH
Fraction Volume: 10 ml

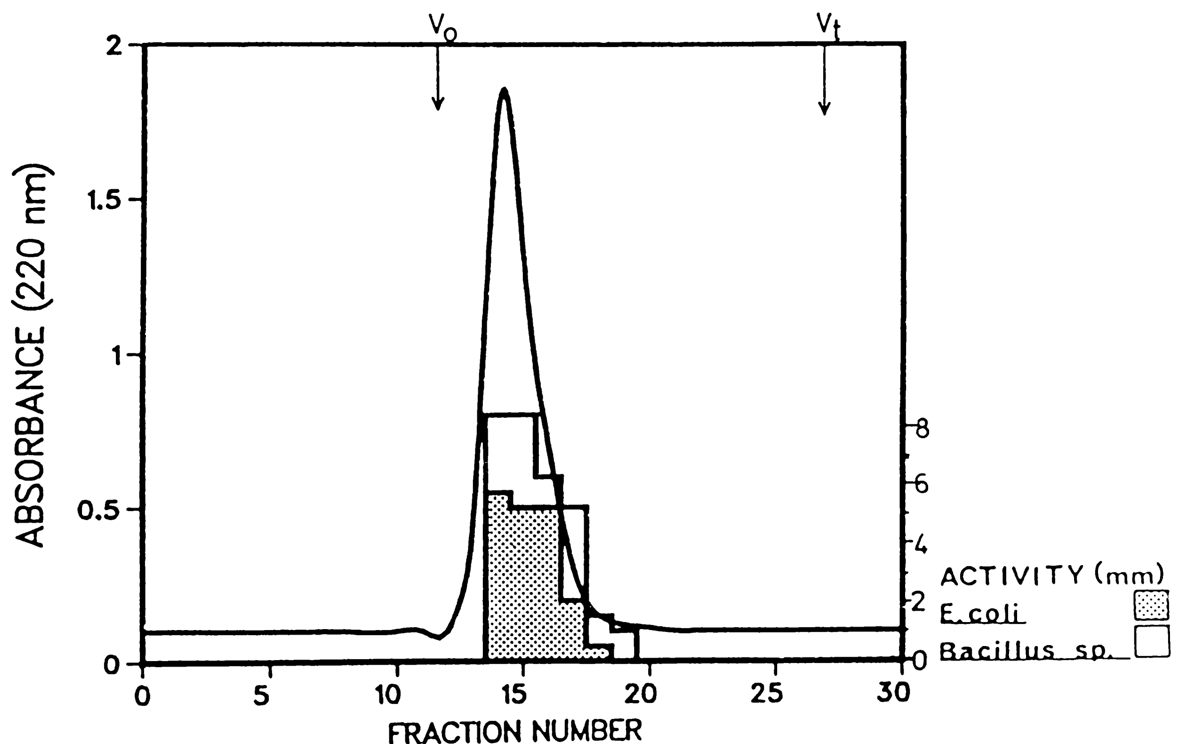


Fig. 6-6: Gel Filtration Chromatography of Fractions 45-52 from Fig. 6-5
Gel: Sephadex G 25 SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 45-52 (Fig. 6-5) evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

in both peaks suggesting either partial insolubility of the material or the slow breaking up of aggregates.

The peak from the wash with NaOH (fractions 45-52) represents the other source of the peptide as isolated by Eschenbruch (1980). Eschenbruch had compared this peptide with that separated earlier in the purification on the Sephadex G 25 column and found them to be identical on cationic electrophoresis. The peak removed by the NaCl from the ion exchange column (Fractions 30-13 in Fig. 6-5) represents lysozyme . These fractions were tested for lytic activity on pregrown *Micrococcus lysodeikticus* plates and were found to be active, whereas the starting thymus preparation had no lysozyme activity. To complete the purification procedure, the lysozyme-containing fractions (13-30) were diluted 1:4 with water and run onto a column of CM Sephadex C 25, washed with 0.05 mol/l phosphate buffer pH 7, and the active fractions were eluted from the column with 0.1 mol/l NaOH.

6.4.2.1 Investigation of the Peptide Fraction from the Component of Higher Molecular Weight

It was evident from the large absorbance obtained from the peptide fraction (fractions 45-52 in Fig. 6-5), that spermine, if present, was combined with a large amount of proteinaceous material. Therefore fractions 45-52 were pooled, evaporated to 5 ml and chromatographed on a Sephadex G 25 column eluted with 0.02 mol/l HCl (pH 1.7) in an attempt to separate any unbound polyamines from the protein. The results are shown in Fig. 6-6. No antibacterial activity was detected in the region of low molecular weight (fractions 22-28) where polyamines would be expected to be eluted from the column. This suggests that either there were no polyamines present or they were bound to the material of high

molecular weight. To investigate whether spermine was present, samples were hydrolysed and run on thin layer chromatography.

6.4.2.2 Investigation of the Spermine Content of the Peptide Fraction from the Component of Higher Molecular Weight

Protein hydrolysis was achieved by refluxing for 18-24 hours in constant-boiling hydrochloric acid. Although this method should have been quite adequate for polyamine extraction, the effectiveness of the hydrolysis conditions was tested. As a control,egg white lysozyme (2 mg) was refluxed with spermine (1 mg) and spermidine (1 mg) for 24 hours in 25 ml of 6 mol/l HCl. The hydrolysate was taken to dryness in a rotary evaporator and washed with distilled water to remove the HCl. Finally, the hydrolysate was dissolved in 50 µl of distilled water and 1 µl of this taken for thin layer chromatography. A 1 ml sample from pooled fractions 13-19 (Fig. 6-6) was freeze-dried and the product (1.4 mg) was hydrolysed using the conditions described above, then run on thin layer chromatography. The results are shown in Fig. 6-7. After spraying with ninhydrin it was found that the lysozyme-polyamine control separated into bands corresponding to spermine, spermidine and amino acids. There appeared to be no interference between the amino acids and the polyamines except that spermidine had a similar R_f value to some of the amino acids, but was visible due to a slight colour difference. The non-hydrolysed egg white lysozyme appeared as a pink spot at the origin. The thymus peptide sample (fractions 13-19, Fig. 6-6) did not appear to have any polyamines present, even when samples were grossly overloaded. The hydrolysed sample appeared to contain a large number of amino acids as it had a similar appearance to the hydrolysed lysozyme.

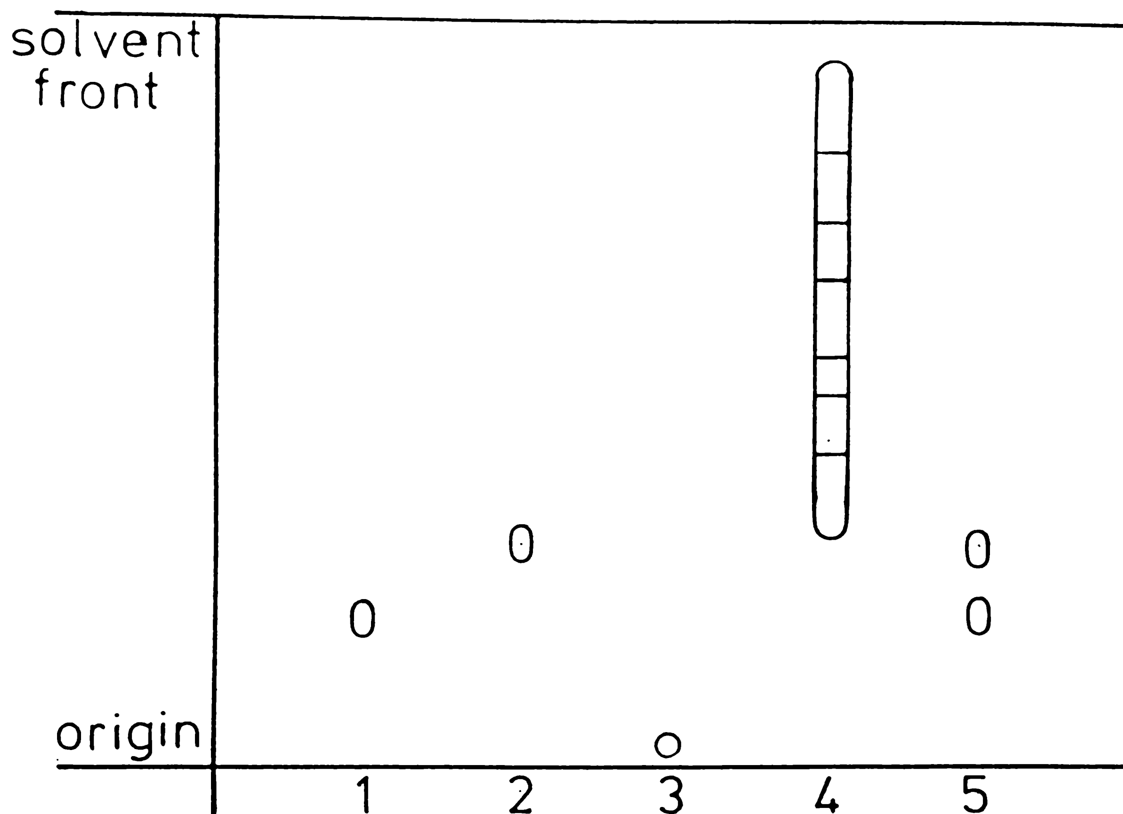


Fig. 6-7: Thin-layer Chromatography
1 - spermine standard
2 - spermidine standard
3 - lysozyme standard
4 - fractions 13-19 from Fig. 6-6
5 - spermine and spermidine standards

6.4.2.3 Attempted Separation of the Antibacterial Compounds from the Peptide Fraction

The peptide fraction from the components of higher molecular weight contained no polyamines and was therefore different from the components of lower molecular weight (Section 6.4.1) which was found to be spermine and spermidine. Further separation of the peptide (fractions 13-19, Fig. 6-6) was attempted, to identify the components present. These fractions were eluted near the void volume of a Sephadex G 25 column. To obtain better resolution they were evaporated to 5 ml and re-chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl. The results are shown in Fig. 6-8. Three broad peaks were obtained (fractions 8-11, 12-18 and 19-22) all of which possessed antibacterial activity. To examine the composition of each peak and the degree of complexity, samples were run on SDS electrophoresis using the method of Laemmli (1970). Samples (1 ml) were taken from fractions 9, 12, 16, 20 and 23, freeze-dried, dissolved in SDS sample buffer and separated by SDS electrophoresis. It was evident from the result (shown in Plate 6-1) that each peak was composed of more than one protein and that further purification would be needed to identify the active components. There were several bands of similar or higher molecular weight than the egg white lysozyme standard, which suggested that lysozyme may have still been present, not having been completely separated by the ion exchange column earlier when following the method of Eschenbruch (1980). Also of interest was the presence of several bands of low molecular weight which may also have been contributing to the overall antibacterial activity.

Plate 6-1: SDS Electrophoresis

1 - lysozyme and trypsinogen standards

2 - lysozyme standard

3 - not relevant

4 - not relevant

5 - not relevant

6 - fraction 9 from Fig. 6-8

7 - fraction 12 from Fig. 6-8

8 - fraction 16 from Fig. 6-8

9 - fraction 20 from Fig. 6-8

10- fraction 23 from Fig. 6-8

Plate 6-2: SDS Electrophoresis

1 - lysozyme and trypsinogen standards

2 - lysozyme standard

3 - not relevant

4 - not relevant

5 - fraction 15 from Fig. 8-16

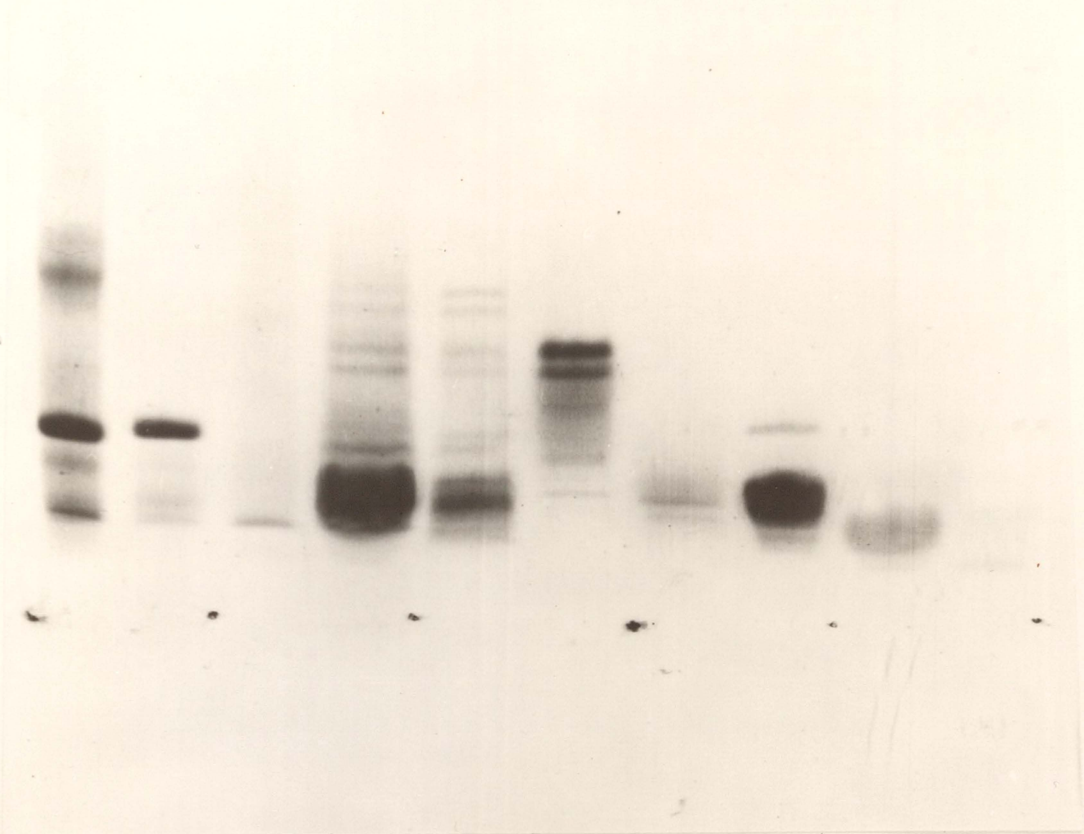
6 - fraction 19 from Fig. 8-16

7 - not relevant

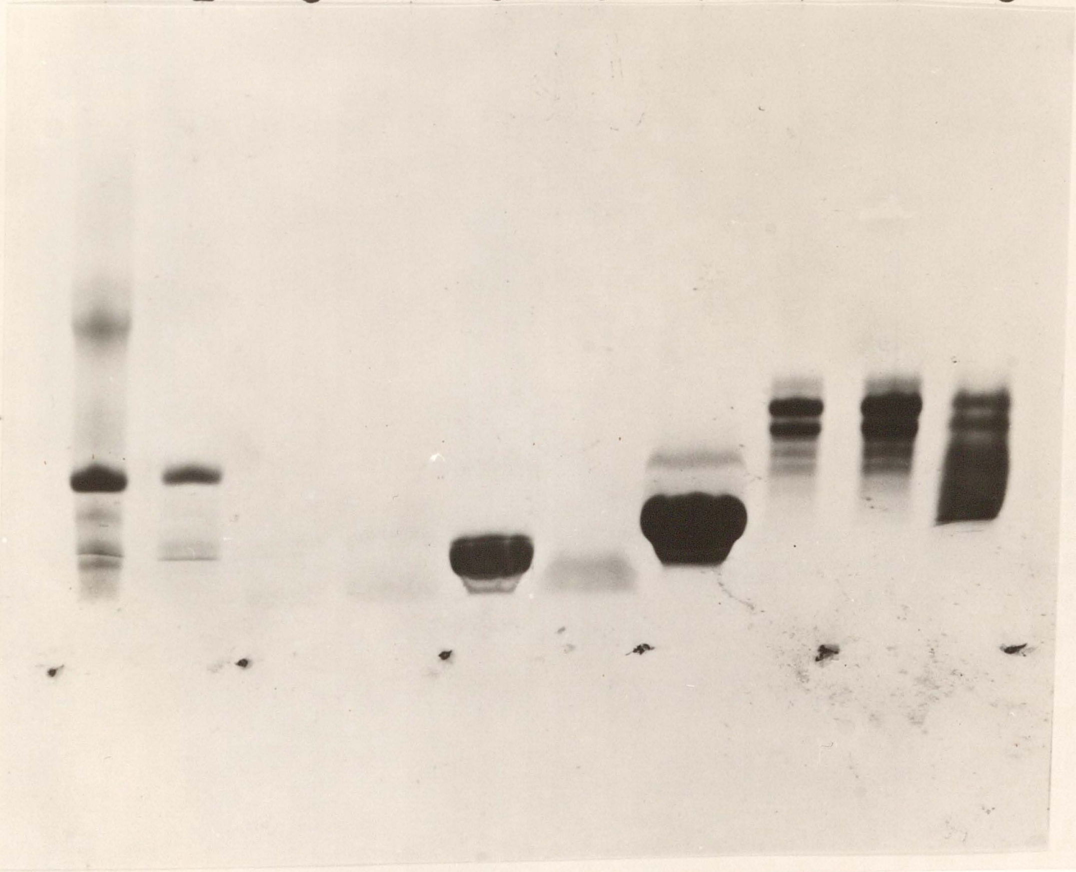
8 - fraction 9 from Fig. 8-12

9 - fraction 9 from Fig. 8-12

10- fraction 12 from Fig. 8-12



1
2
3
4
5
6
7
8
9
10
−
+



1
2
3
4
5
6
7
8
9
10
−
+

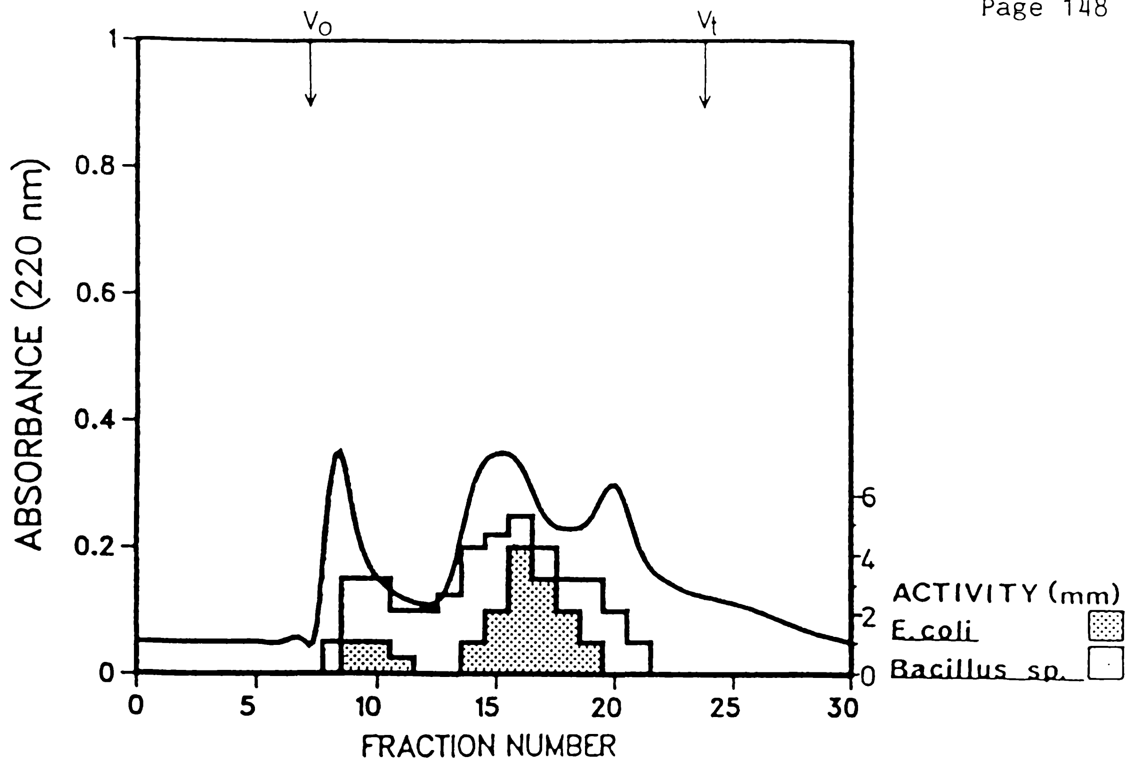


Fig. 6-8: Gel Filtration Chromatography of Fractions 13-19 from Fig. 6-6
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 13-19 (Fig. 6-6) evaporated to 5 ml
Fraction Volume: 10 ml

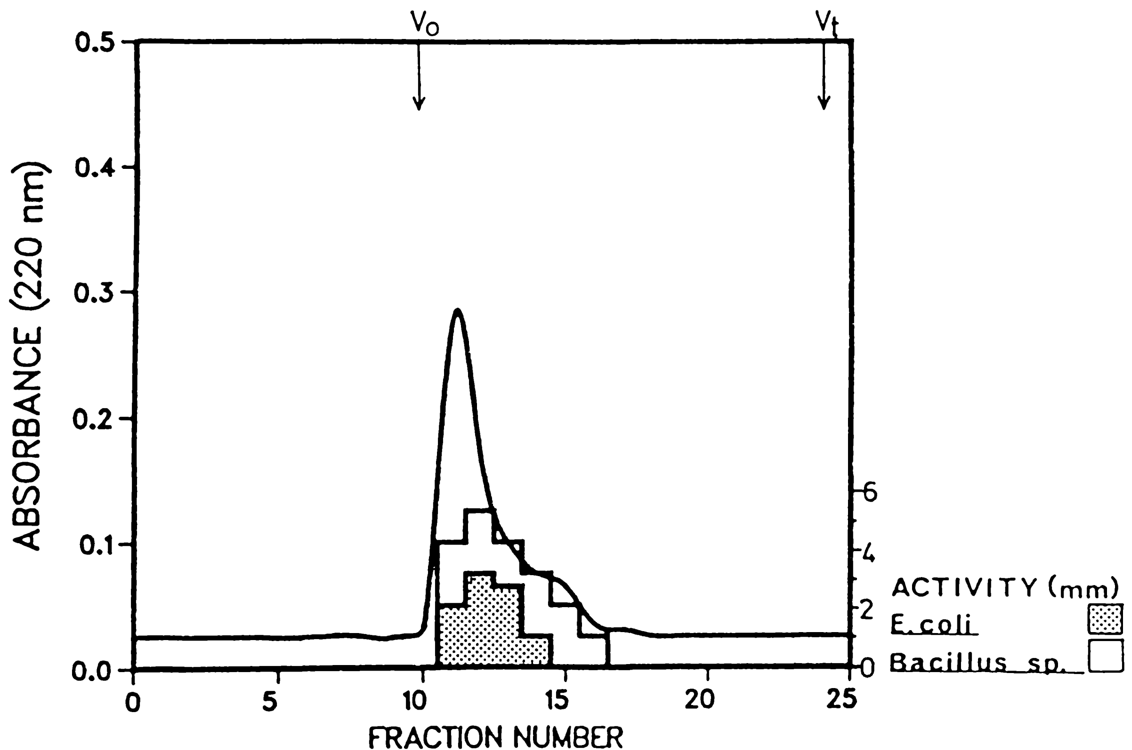


Fig. 6-9: Gel Filtration Chromatography of Fractions 8-11 from Fig. 6-8
Gel: Sephadex G 50 F
Eluent: 0.01 mol/l NaOH, pH 12
Sample: Fractions 8-11 (Fig. 6-8) evaporated to 5 ml and adjusted to pH 12
Fraction Volume: 10 ml

6.4.2.3.1 Separation of Fractions 8-11

In an attempt to separate the components of fractions 8-11 (Fig. 6-8) they were chromatographed under conditions of high pH to enhance disaggregation. Fractions 8-11 were evaporated to 5 ml, adjusted to pH 12 by the addition of 1 mol/l NaOH and chromatographed through a column of Sephadex G 50 eluted with 0.01 mol/l NaOH pH 12. The fractions were neutralised as they were eluted from the column and assayed for antibacterial activity. The results are shown in Fig. 6-9. Activity was found to correspond with the peak of absorbance. The use of high pH appears to have been of little use in disaggregating the antibacterial compounds. SDS electrophoresis of Fraction 9 (Plate 6-1) demonstrated that it contained components with a range of molecular weights from approximately 10 000 to 20 000. The active fractions were eluted near the void volume of the column of Sephadex G 50, thus apparently having molecular weights of approximately 40 000-50 000. This suggested that aggregation was occurring during gel filtration chromatography. In an attempt to break down these aggregates, an eluent of low pH was used. Fractions 11-16 (Fig. 6-9) were evaporated to 5 ml and re-chromatographed on a column of Sephadex G 75 eluted with 0.02 mol/l HCl (pH 1.7). The results are shown in Fig. 6-10. The large broad peak had antibacterial activity and again there appeared that little separation had been achieved.

Ion exchange chromatography was again used in an attempt to separate these compounds. Fractions 8-14 (Fig. 6-10) were adjusted to pH 7 and run onto a column of CM Sephadex C 25 equilibrated with 0.1 mol/l phosphate buffer pH 7. The column was eluted with a salt gradient from 0 to 2.0 mol/l NaCl followed by a final wash with 0.1 mol/l NaOH. The results are shown in Fig. 6-11. The active

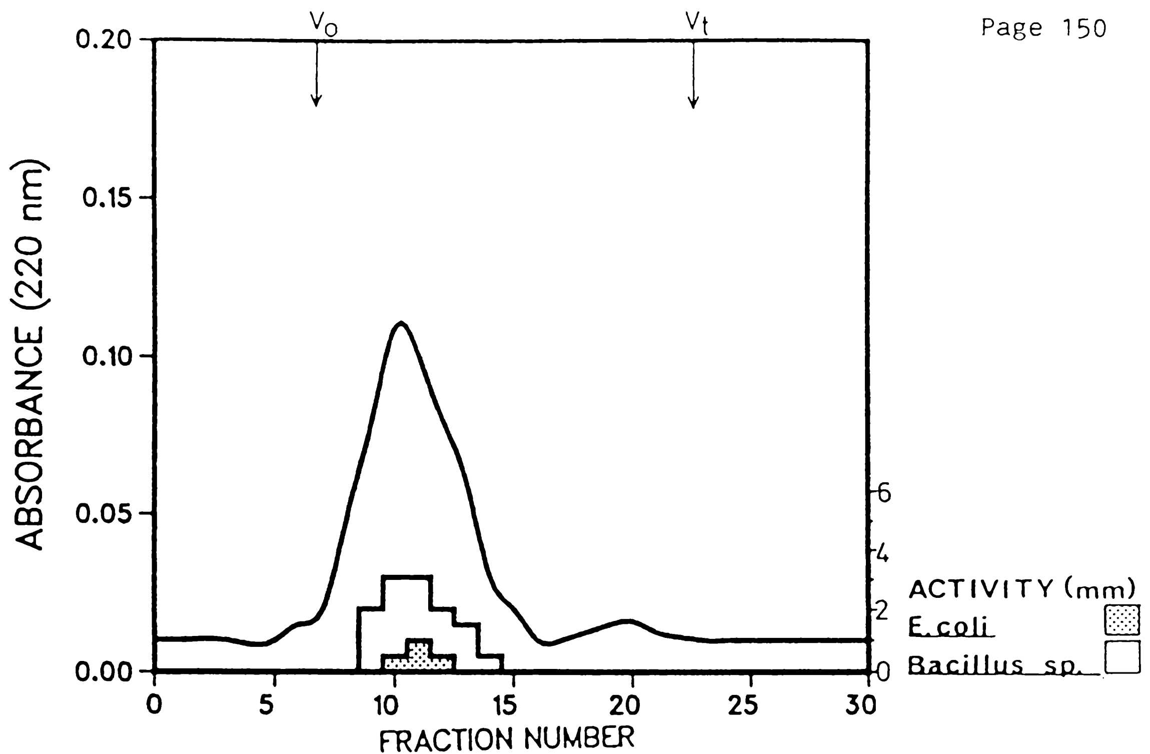


Fig. 6-10: Gel Filtration Chromatography of Fractions 11-16 from Fig. 6-9
Gel: Sephadex G 75 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 11-16 (Fig. 6-9) evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

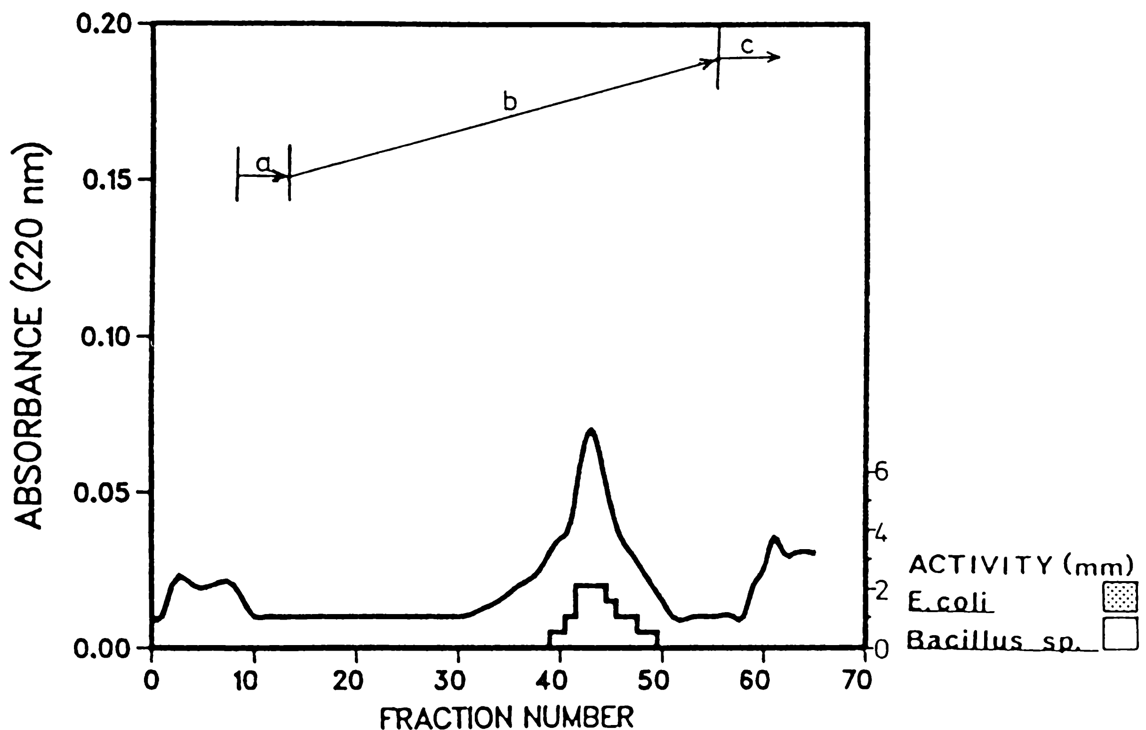


Fig. 6-11: Ion Exchange Chromatography of Fractions 8-14 from Fig. 6-10
Gel: CM Sephadex C 25
Buffer: 0.1 mol/l phosphate, pH 7
Sample: Fractions 8-11 (Fig. 6-10) adjusted to pH 7
Elution Buffers: a- 0.1 mol/l phosphate, pH 7; b- salt gradient from 0 to 2 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 7; c- 0.1 mol/l NaOH
Fraction Volume: 10 ml

material was eluted from the column with the salt gradient in a single broad peak. To concentrate the active fractions and to remove the salt they were diluted 1:1 with water, re-run through the column of CM Sephadex C 25, washed with 0.05 mol/l phosphate buffer pH 7, and eluted with 0.1 mol/l NaOH. The fractions eluted were adjusted to pH 1.7, evaporated to 5 ml and chromatographed through a column of Sephadex G 50 eluted with 0.02 mol/l HCl. The results are shown in Fig. 6-12. A single active peak (fractions 8-12) was eluted near the void volume. Samples of 1 ml were taken from fractions 9 and 12 (shown in Fig.-12), freeze-dried, re-dissolved in SDS sample buffer and separated by SDS electrophoresis. The results are shown in Plate 6-2. Fractions 9 and 12 were found to be still heterogeneous, the gel filtration and ion exchange chromatography using the various eluents not separating out the active components. Again it appeared that aggregation of these compounds was occurring: the range of molecular weights found on SDS electrophoresis was approximately 10 000-20 000 compared with the value of 30 000 suggested by elution near the void volume of the column of Sephadex G 50. As this problem may have been associated only with the components of higher molecular weight, separation of the other components of lower molecular weight was attempted.

6.4.2.3.2 Separation of Fractions 12-18

Fractions 12-18 shown in Fig. 6-8 were adjusted to pH 7 and run on a column of CM Sephadex C 25 equilibrated with 0.1 mol/l phosphate buffer pH 7. The column was eluted with a salt gradient from 0 to 1 mol/l NaCl in 0.1 mol/l phosphate buffer pH 7 followed by a wash with 1 mol/l NaCl in 0.1 mol/l phosphate buffer pH 7. Any remaining material was removed by a final wash with 0.1 mol/l NaOH. The results are shown

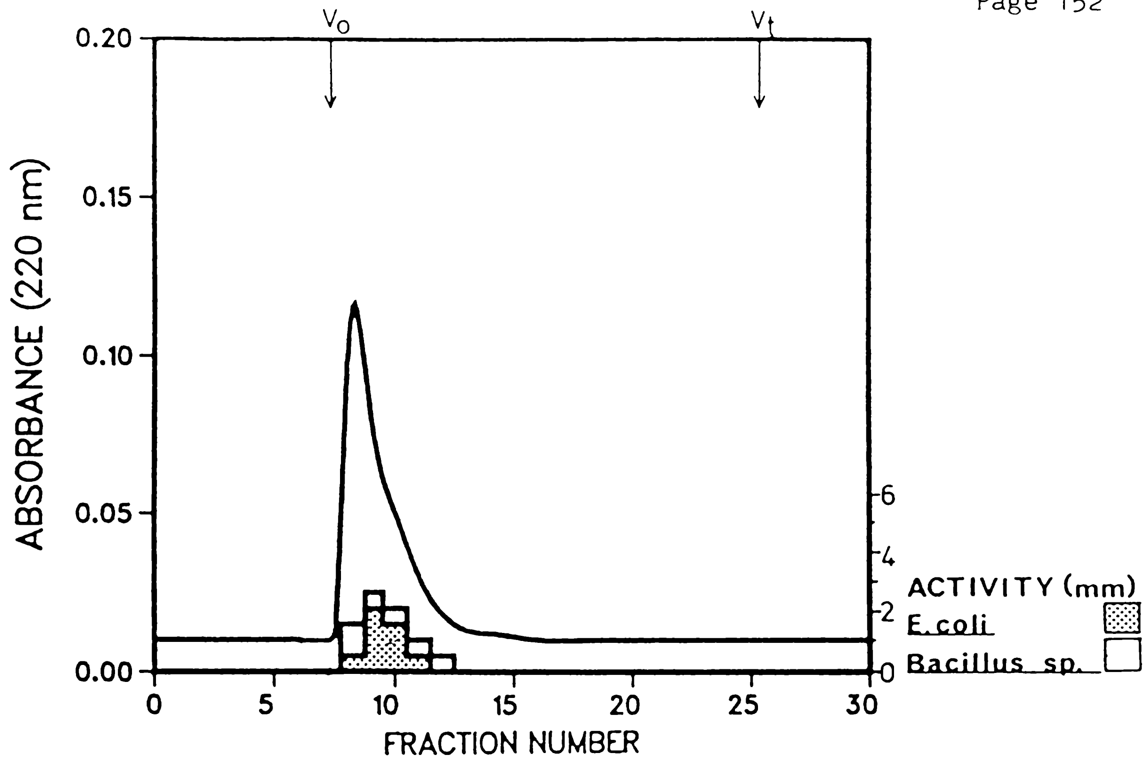


Fig. 6-12: Gel Filtration Chromatography on the Active Fraction from Fig. 6-11
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Active fractions (Fig. 6-11), desalted, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

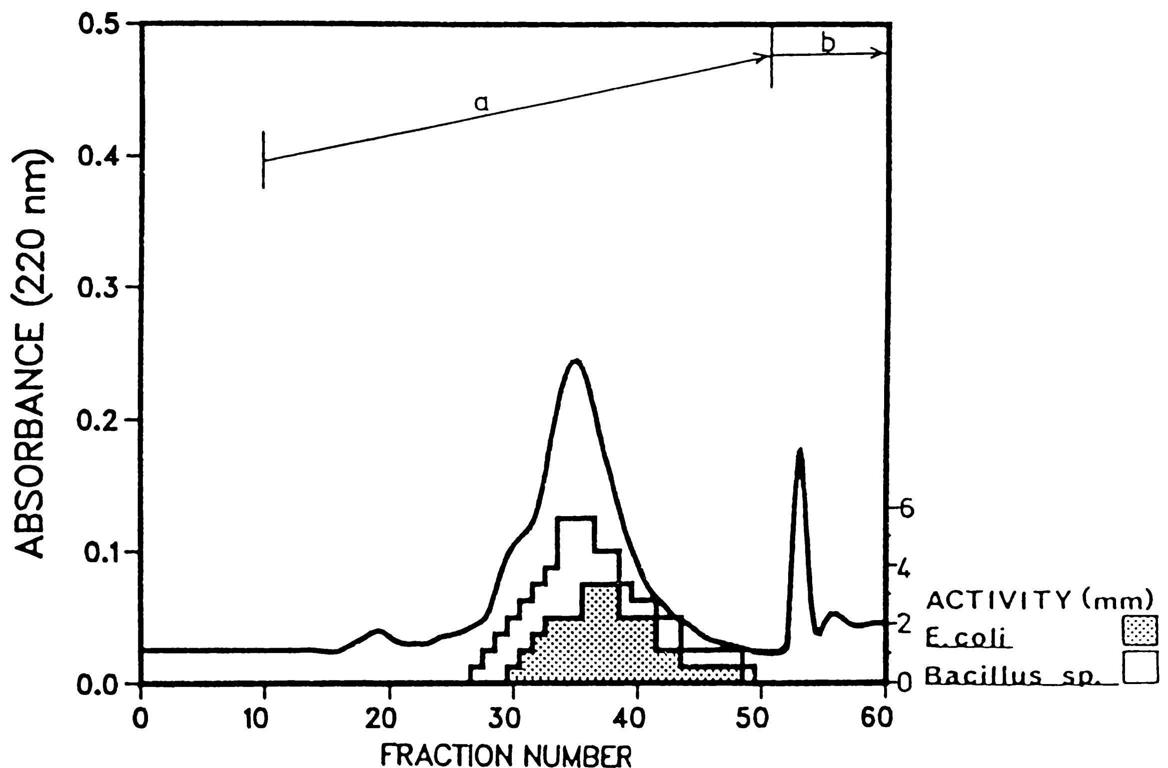


Fig. 6-13: Ion Exchange Chromatography of Fraction 12-18 from Fig. 6-8
Gel: CM Sephadex C 25
Buffer: 0.1 mol/l phosphate, pH 7
Sample: Fractions 12-18 (Fig. 6-8) adjusted to pH 7
Elution Buffers: a- salt gradient from 0 to 1 mol/l NaCl in 0.1 mol/l phosphate, pH 7; b- 0.1 mol/l NaOH
Fraction Volume: 10 ml

in Fig. 6-13. The antibacterial activity was eluted off the ion exchanger by the salt gradient in a similar position as in Fig. 6-11. To concentrate and remove the salt the active fractions (26-48 in Fig. 6-13) were diluted 1:1 with distilled water and re-run through the column of CM Sephadex C 25, washed with 0.05 mol/l phosphate buffer pH 7, and the active fractions eluted with 0.1 mol/l NaOH. These fractions were immediately neutralised, evaporated to 5 ml and chromatographed on a column of Sephadex G 25 eluted with distilled water. Distilled water was used as an eluent to make use of the gel-protein interactions common with eluents of low ionic strength as an aid in separating the antibacterial components. The results are shown in Fig. 6-14. A peak was resolved which had two broad shoulders. As the first shoulder came out near the void volume of the Sephadex G 25, fractions 10-17 were evaporated to 5 ml and re-chromatographed on a column of Sephadex G 50 eluted with distilled water. However, the active material came out as a single peak near to the bed volume of the Sephadex G 50 as shown in Fig. 6-15. A 1 ml sample from fraction 23 (Fig. 6-15) was freeze-dried, re-dissolved in SDS sample buffer, and run on SDS electrophoresis (Plate 6-3). There was a complex pattern of bands present ranging from some of high molecular weight (approximately 30 000) to the major band in the low molecular weight region (approximately 10 000). The estimated molecular weight of the peak from the elution volume of the column was approximately 5 000, which is lower than the range of molecular weights found on SDS electrophoresis. This suggested that gel-protein interaction was occurring during column chromatography causing this complex mixture of proteins to be eluted near the bed volume of the column of Sephadex G 50. These proteins were difficult to separate using conventional chromatographic methods. This complex antibacterial material was left at this stage and attention was

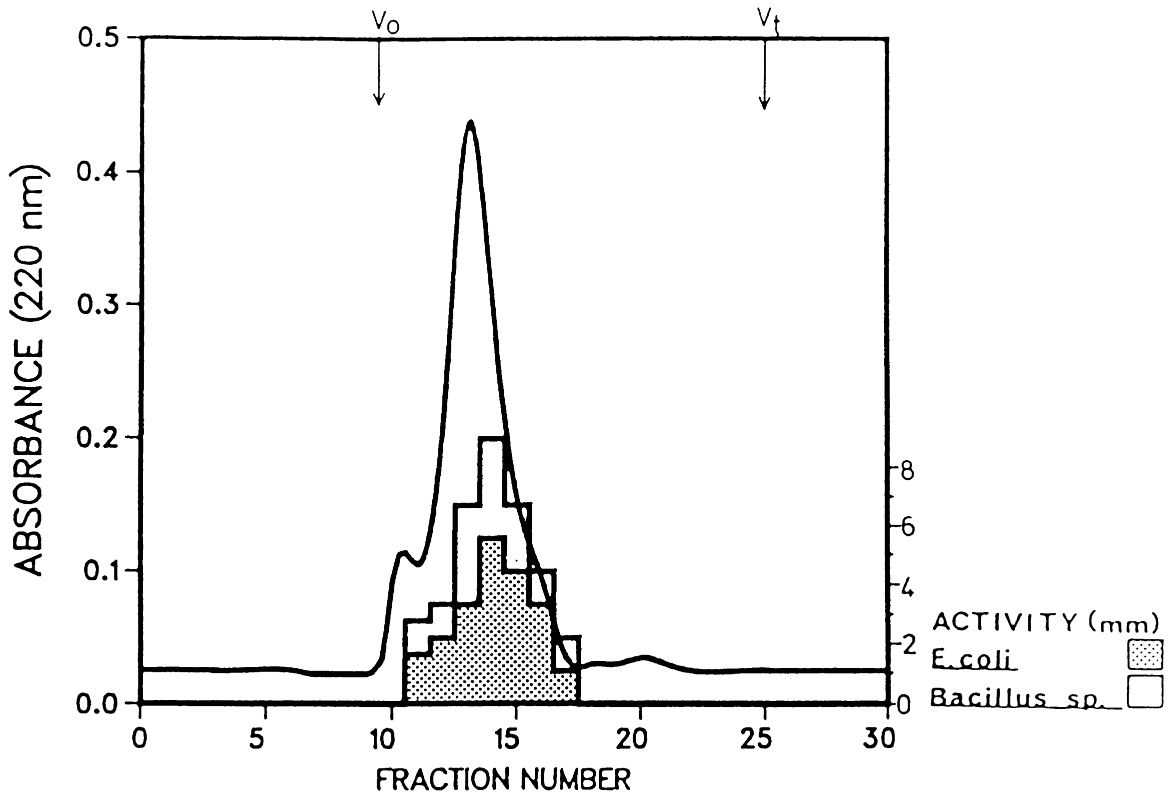


Fig. 6-14: Gel Filtration Chromatography of the Active Fractions from Fig. 6-13
Gel: Sephadex G 25 SF
Eluent: Distilled water
Sample: Active fractions (Fig. 6-13), desalted and evaporated to 5 ml
Fraction Volume: 10 ml

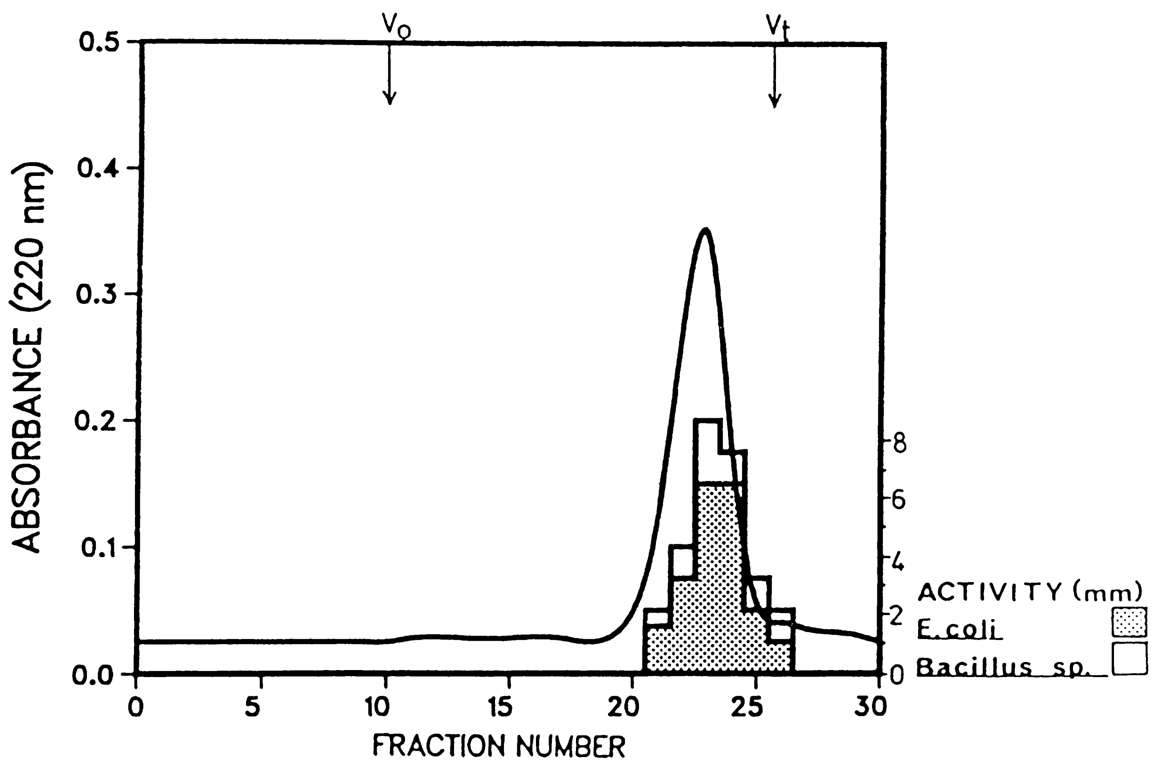


Fig. 6-15: Gel Filtration Chromatography of Fractions 10-17 from Fig. 6-14
Gel: Sephadex G 50 F
Eluent: Distilled water
Sample: Fractions 10-17 (Fig. 6-14) evaporated to 5 ml
Fraction Volume: 10 ml

Plate 6-3: SDS Electrophoresis

1 - fraction 23 from Fig. 6-15

2 - fraction 23 from Fig. 6-15

3 - fraction 18 from Fig. 6-19

4 - fraction 14 from Fig. 6-19

5 - fraction 14 from Fig. 6-20

6 - lysozyme and trypsinogen standards

Plate 6-4: SDS Electrophoresis

1 - lysozyme standard

2 - not relevant

3 - fraction 14 from Fig. 6-21

4 - fraction 14 from Fig. 6-21

5 - fraction 18 from Fig. 6-21

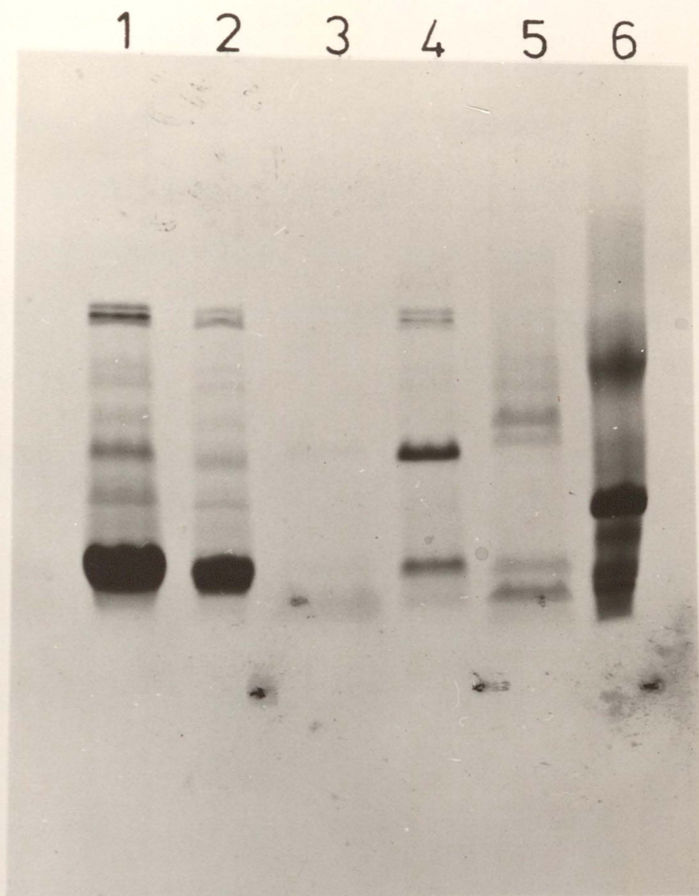
6 - fraction 18 from Fig. 6-21

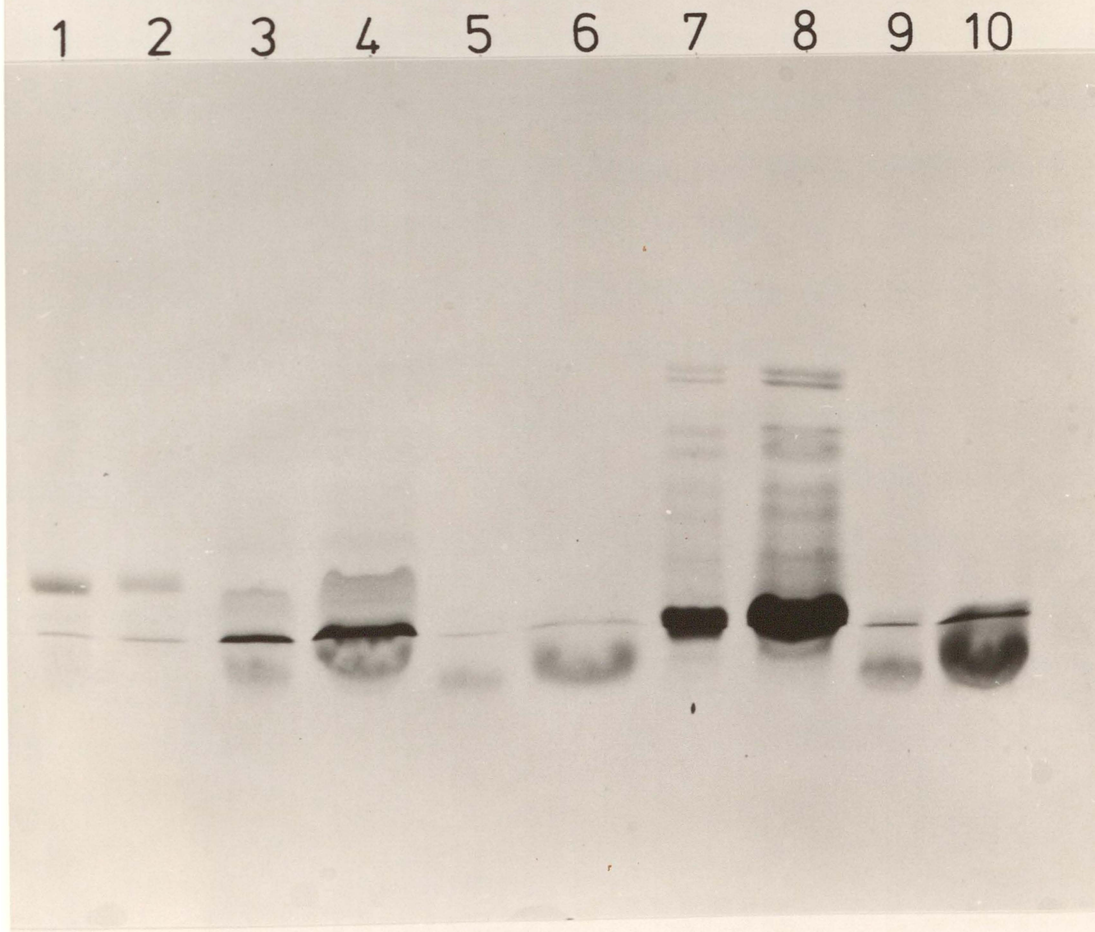
7 - fraction 14 from Fig. 6-22

8 - fraction 14 from Fig. 6-22

9 - fraction 18 from Fig. 6-22

10- fraction 18 from Fig. 6-22


1 2 3 4 5 6
−
+


1 2 3 4 5 6 7 8 9 10
−
+

turned to the last remaining active peak shown in Fig. 6-8 (fractions 19-22). SDS electrophoresis of fraction 20 (Fig. 6-8) shown in Plate 6-1 indicated the presence of one broad band of high mobility. Therefore fractions 19-22 may have been less complex and hence the active component(s) not as difficult to isolate as those in fractions 8-11 and 12-18.

6.4.2.3.3 Separation of Fractions 19-22

Fractions 19-22 (Fig. 6-8) had been eluted near the bed volume of the Sephadex G 50 column so they were evaporated and chromatographed through a column of Sephadex G 25 eluted with 0.02 mol/l HCl, pH 1.7. However the active fractions were eluted from the Sephadex G 25 near the void volume as shown in Fig. 6-16. Samples of 1 ml were taken from fractions 15 and 19, freeze-dried, re-dissolved in SDS sample buffer and examined by SDS electrophoresis. It was evident that a major low molecular weight peak predominated (see Plate 6-2). Fractions 14-21 (Fig. 6-16) were adjusted to pH 7 and run onto a column of CM Sephadex C 25 equilibrated with 0.1 mol/l phosphate buffer pH 7. The column was eluted with a salt gradient from 0 to 1.0 mol/l NaCl in 0.1 mol/l phosphate buffer pH 7 followed by a wash with 1.0 mol/l NaCl in 0.1 mol/l phosphate buffer pH 7. All remaining material was removed by a final wash with 0.1 mol/l NaOH. The results are shown in Fig. 6-17. This time the ion exchange column appeared to separate a lot of inactive protein during the salt gradient. However the active fractions were eluted from the column in a position similar to that found (Fig. 6-13). There was also an additional active peak washed off with 1.0 mol/l NaCl. This however may have been the tail end of the activity washed off by the salt gradient.

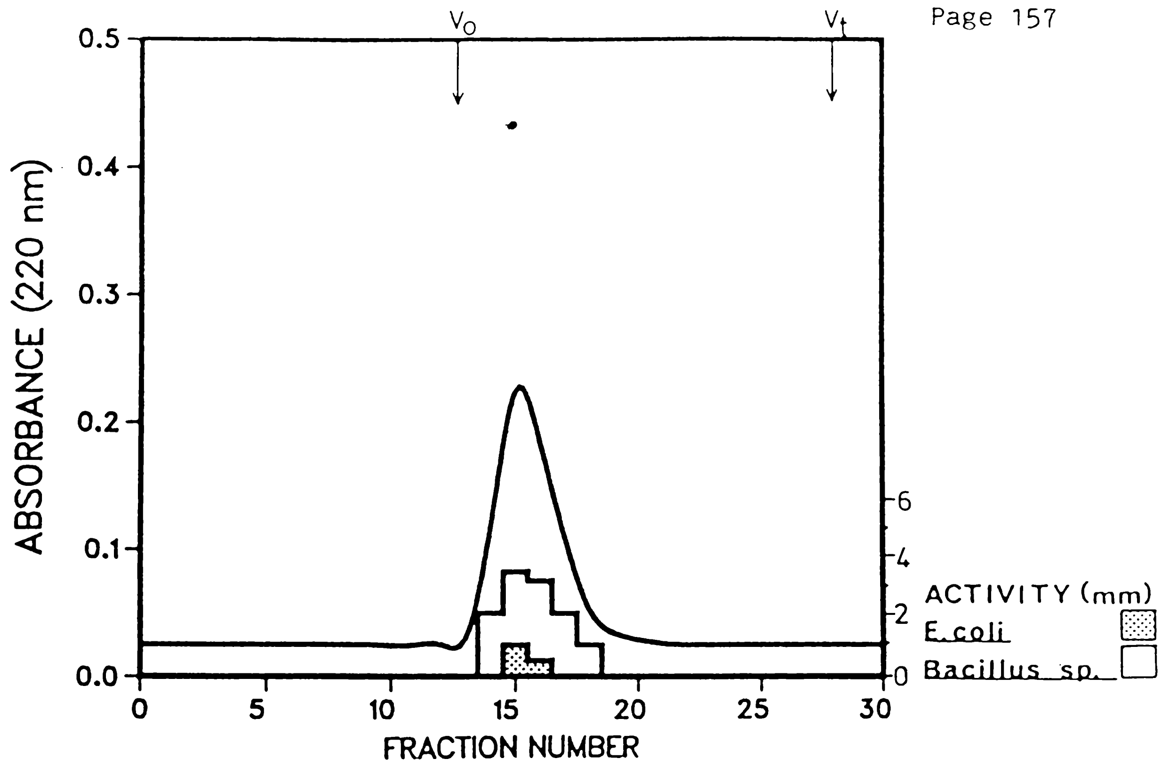


Fig. 6-16: Gel Filtration Chromatography of Fractions 19-22 from Fig. 6-8
Gel: Sephadex G 25 SF
Eluent: 0.02 mol/l HCl, pH 7
Sample: Fractions 19-22 (Fig. 6-8) evaporated to 5 ml
Fraction Volume: 10 ml

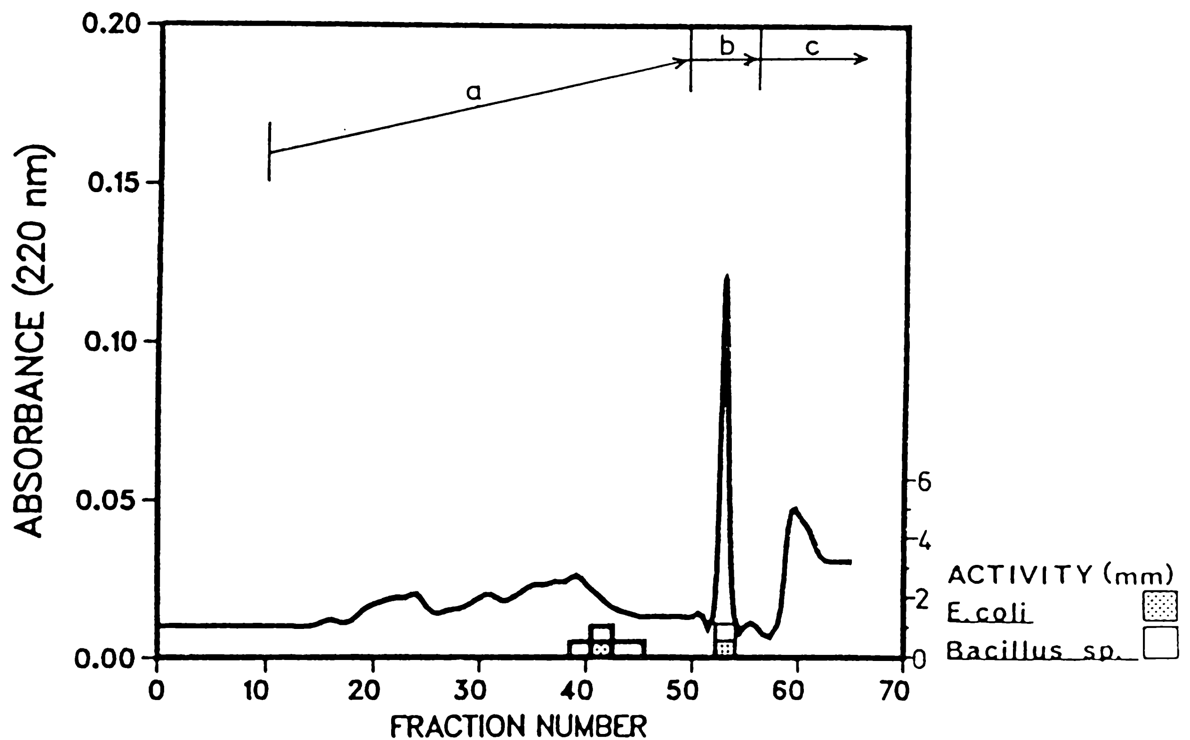


Fig. 6-17: Ion Exchange Chromatography of Fractions 14-21 from Fig. 6-16
Gel: CM Sephadex C 25
Buffer: 0.1 mol/l phosphate, pH 7
Sample: Fractions 14-21 (Fig. 6-16) adjusted to pH 7
Elution Buffers: a- 0.1 mol/l phosphate, pH 7; b- salt gradient from 0 to 1 mol/l NaCl in phosphate buffer, pH 7; c- 0.1 mol/l NaOH
Fraction Volume: 10 ml

Fractions 35-49 were diluted 1:1 with distilled water and re-run onto the column of CM Sephadex C 25, washed with 0.05 mol/l phosphate buffer pH 7, and eluted with 0.1 mol/l NaOH. These fractions were immediately neutralised, evaporated to 5 ml and chromatographed on a column of Sephadex G 50 eluted with distilled water. The resulting broad elution profile (Fig. 6-18) indicated that two peaks were eluted close together near to the bed volume of the Sephadex G 50. In order to obtain better separation, fractions 19-27 were evaporated to 5 ml and re-chromatographed on a column of Sephadex G 25 eluted with 0.02 mol/l HCl. Unfortunately the protein was eluted at the void volume of the Sephadex G 25 (Fig. 6-19).

Fractions 14 and 18 (Fig. 6-19) were freeze-dried, re-dissolved in SDS sample buffer and examined by SDS electrophoresis. Fraction 14, which was the most active fraction, had at least 5 visible bands of which 4 were very distinct (see Plate 6-3).

Fractions 53-54 from the ion exchange chromatography (shown in Fig. 6-17) were evaporated to 10 ml, adjusted to pH 1.7 with HCl and chromatographed on a column of Sephadex G 25 eluted with 0.02 mol/l HCl (Fig. 6-20). Fraction 14 was the only one with activity, probably because there was a small amount of protein present in the other fractions. A 2 ml sample from fraction 14 was freeze-dried, re-dissolved in SDS sample buffer, and run on SDS electrophoresis. The results are shown in Plate 6-3. The electrophoresis showed that there were still several bands present in fraction 14 (Fig. 6-20) and only one minor band corresponded with the bands in fraction 14 (Fig. 6-19). This may be the active component, which would explain why this fraction was not very active. In comparing the SDS electrophoresis patterns of fraction 20 (shown in Plate 6-1) and fractions 14 (shown in Plate 6-3),

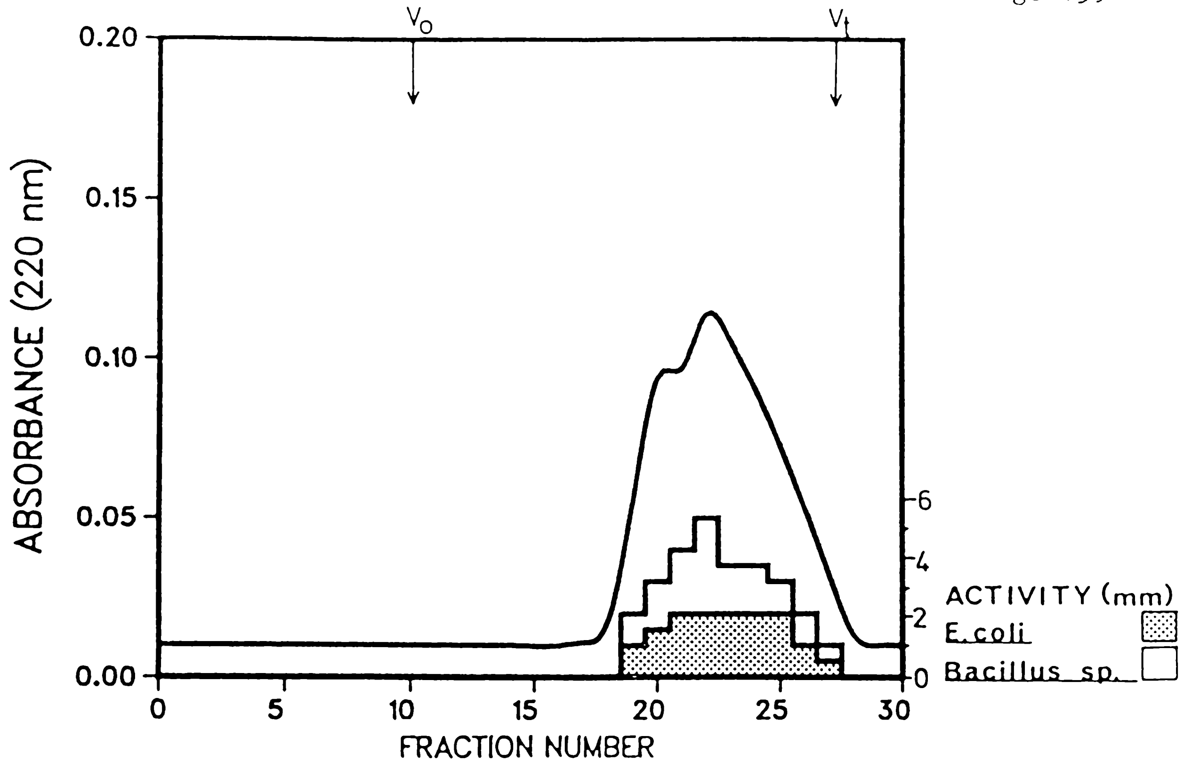


Fig. 6-18: Gel Filtration Chromatography of Fractions 35-49 from Fig. 6-17
Gel: CM Sephadex C 25
Eluent: Distilled water
Sample: Fractions 35-49 (Fig 6-17), desalted and evaporated to 5 ml
Fraction Volume: 10 ml

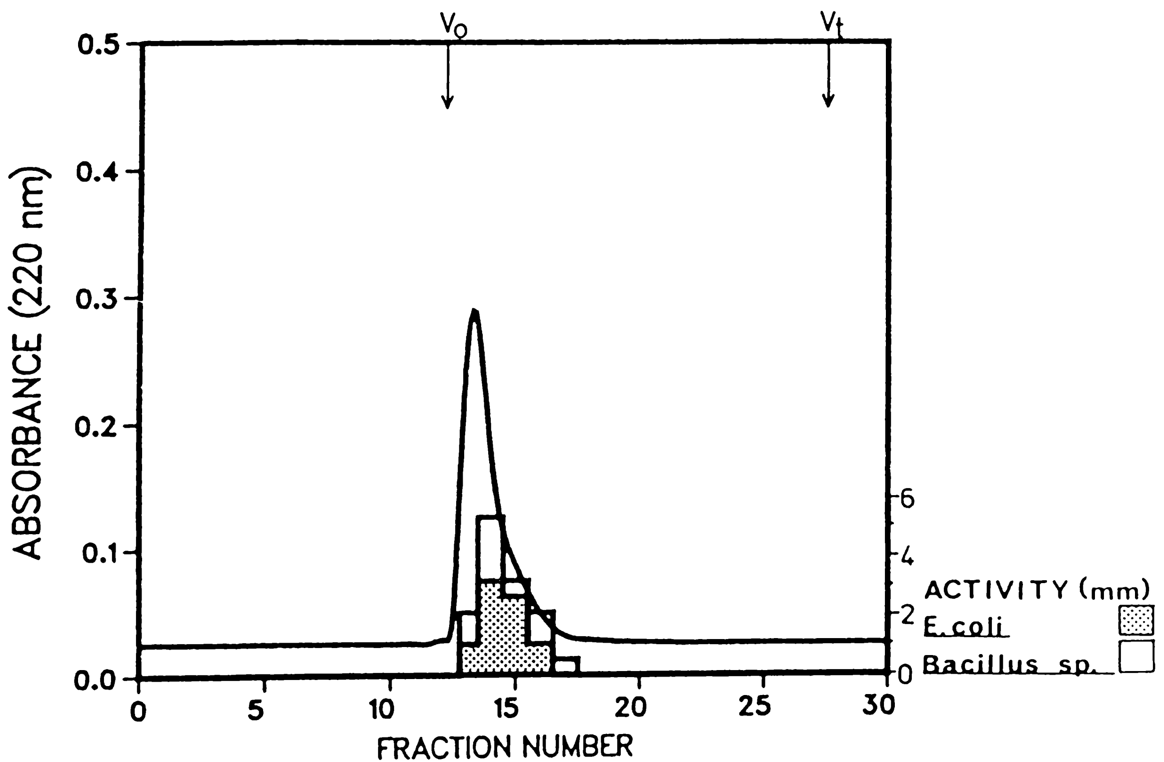


Fig. 6-19: Gel Filtration Chromatography of Fractions 19-27 from Fig. 6-18
Gel: Sephadex G 50 F
Eluent: Distilled water
Sample: Fractions 19-27 (Fig. 6-18) evaporated to 5 ml
Fraction Volume: 10 ml

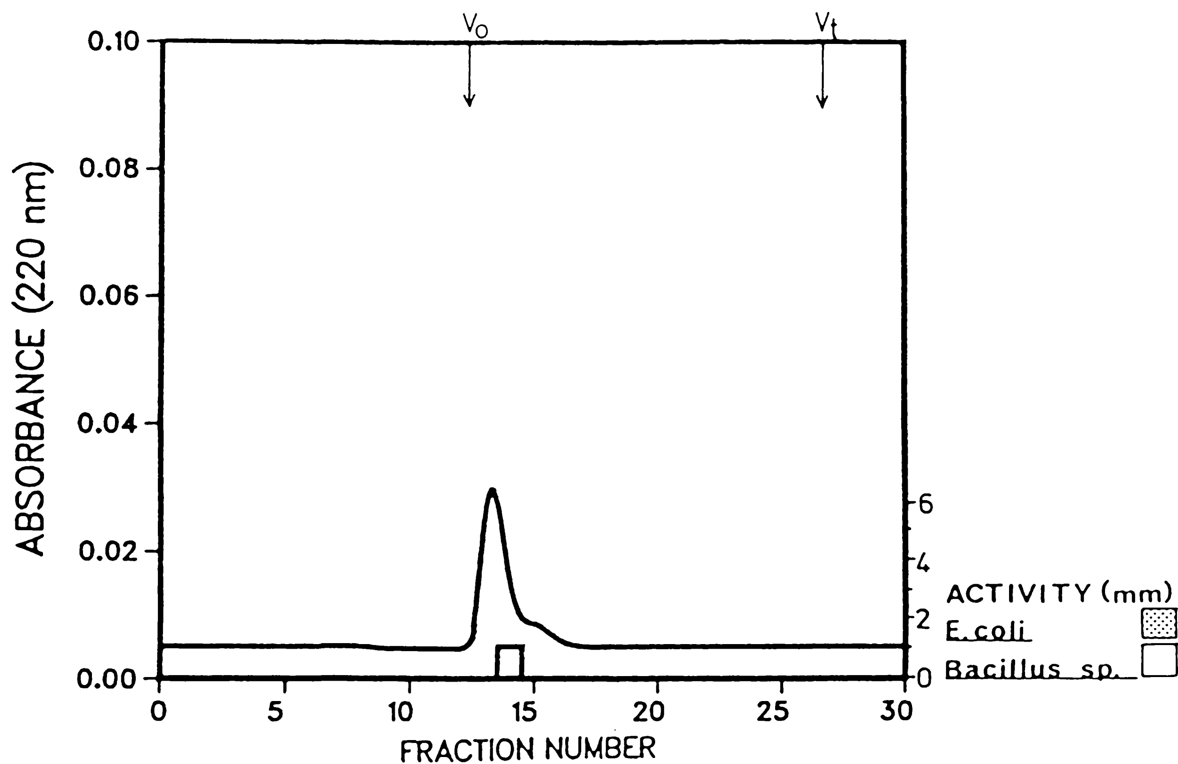


Fig. 6-20: Gel Filtration Chromatography of Fractions 53-54 from Fig. 6-17
Gel: Sephadex G 25 SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 53-54 (Fig. 6-17) evaporated to 10 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

it was noted that a number of bands of higher molecular weight arose from chromatography of fractions 19-22.

The methods of gel filtration and ion exchange chromatography seem to be unable to separate these aggregated compounds. This was further demonstrated when the lysozyme fraction from Fig. 6-5 (fractions 13-30) was also found to be highly complex when run on gel filtration chromatography and examined on SDS electrophoresis (not shown).

Conclusions

The results obtained following the purification method outlined by Eschenbruch (1980) were markedly different from those originally obtained. Eschenbruch isolated two antibacterial compounds from the thymus preparation (Section 6.3), lysozyme and a peptide. In the present study the peptide was first isolated from the column of Sephadex G 25 (fractions 22-25) and subsequently identified as a mixture of spermine and spermidine (section 6.4.1). Eschenbruch further purified fractions 14-20 by ion exchange chromatography to yield lysozyme (eluted with 0.4 mol/l NaCl) and more peptide (eluted with 0.1 mol/l NaOH). Eschenbruch found the two peptide fractions to have similar mobilities on cationic electrophoresis. However, in the present study the peptide fraction from the ion exchange chromatography did not contain spermine or spermidine, and appeared to be a complex mixture of peptides and proteins. The lysozyme fraction from the ion exchange chromatography was also of a different composition from that found by Eschenbruch. However with such extracts result are often very difficult to repeat. In order to eliminate the possibility that a major difference resulted during the purification, the procedures were repeated. The sheep thymus peptide was isolated according to Dubos and Hirsch (1954) and the

purification of lysozyme and the thymus peptide was attempted. However, similar results were obtained: only complex heterogeneous compounds were isolated.

As there appeared to be antibacterial peptides present which were not spermine or spermidine, it was decided to investigate these peptides and to compare them with other isolated proteins such as those from milk cells, seminalplasmin and beta-lysin.

6.5 Further Attempts at Isolating the Antibacterial Compounds from the Sheep Thymus Preparation

The sheep thymus peptide was extracted according to the method of Dubos and Hirsch (1954). The thymus peptide (100 mg) was dissolved in 5 ml of 0.02 mol/l HCl and chromatographed through a column of Sephadex G 25 eluted with 0.02 mol/l HCl pH 1.7 (as shown in Fig. 6-1) to remove the spermine and spermidine. The polyamine fractions (25-26) were separated from the other active fractions and were stored. Fractions 14-22 were pooled, evaporated to near dryness and re-suspended in 4.5 mol/l urea. The urea was added in an attempt to achieve disaggregation of the antibacterial components. The solution was then run onto a column of CM Sephadex C 25 equilibrated with 4.5 mol/l urea in 0.1 mol/l imidazole buffer pH 7. A salt gradient from 0 to 1 mol/l NaCl in imidazole-urea buffer, pH 7, was run through the column, followed by a wash of 0.1 mol/l phosphate buffer, pH 7, and finally the column was eluted with 0.1 mol/l NaOH to remove any remaining protein. The results are shown in Fig. 6-21. Four areas of absorbance were detected, fractions 2-6, 18-28, 29-42 and 56-58. These fractions were pooled in these groups, diluted 1:2 with distilled water and adjusted to pH 6 then run onto a column of CM Sephadex C 25 equilibrated with 0.1 mol/l

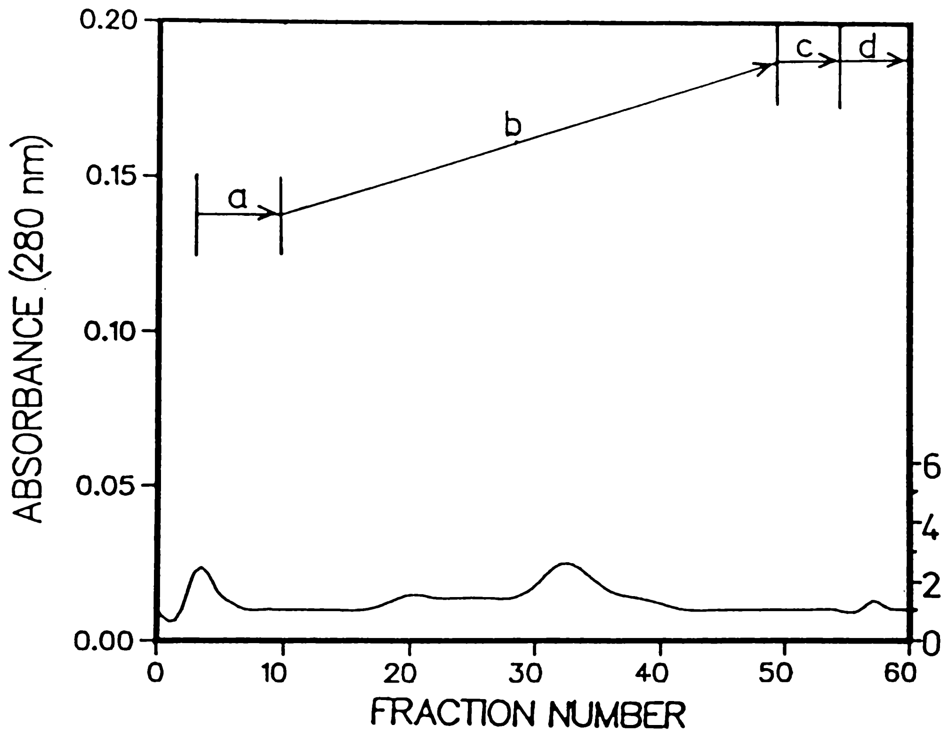


Fig. 6-21: Ion Exchange Chromatography of Fractions 14-22 from Fig. 6-1
Gel: CM Sephadex C 25
Buffer: 4.5 mol/l urea in 0.1 mol/l imidazole, pH 7
Sample: Fractions 14-22 (Fig. 6-1) evaporated to near dryness and re-dissolved in urea/imidazole buffer, pH 7
Elution Buffers: a- urea/imidazole buffer, pH 7; b- salt gradient from 0 to 1 mol/l NaCl in urea/imidazole buffer, pH 7; c- 0.1 mol/l phosphate buffer, pH 7; d- 0.1 mol/l NaOH
Fraction Volume: 10 ml

Phosphate buffer, pH 6 to remove the salt and urea and to concentrate the fractions. The column was washed with 0.1 mol/l phosphate buffer, pH 6, and the protein removed with 0.1 mol/l NaOH. The eluted fractions were neutralised and assayed for antibacterial activity. After this treatment pooled fractions 18-28 and 29-42 both displayed antibacterial activity against E. coli and the Bacillus species. The other two pools were inactive.

The two active pools were each evaporated to 5 ml and re-chromatographed on a column of Sephadex G 25 eluted with 0.02 mol/l HCl. The results are shown in Fig. 6-22a and 6-22b. Both runs gave broad peaks with activity spread throughout. The urea appears to have had little effect in disaggregating these complexes (compare Fig. 6-22a with Fig. 6-6) Samples (0.5 ml) were taken from fractions 14 and 18 from the chromatography shown in Figs. 6-22a and 6-22b, freeze-dried, re-dissolved in SDS sample buffer and separated by SDS electrophoresis. The results are shown in Plate 6-4. There were multiple bands present in fraction 14 from both runs, and they appeared to be similar. There appeared to be a greater number of bands of high molecular weight from fraction 14 (Fig. 6-22b) although those from fraction 14 (Fig. 6-22a) were less obvious because of the lower protein concentration overall. As a further comparison these same fractions were run on cationic electrophoresis. The results are shown in Plate 6-5. Again multiple bands were present in each sample. It is interesting to note that fraction 18 of both runs ran as a single band on SDS electrophoresis, a dissociating technique, and as multiple bands on cationic electrophoresis. The multiple bands may have been due to aggregation occurring on cationic electrophoresis or compounds of similar molecular weight separating according to their charge.

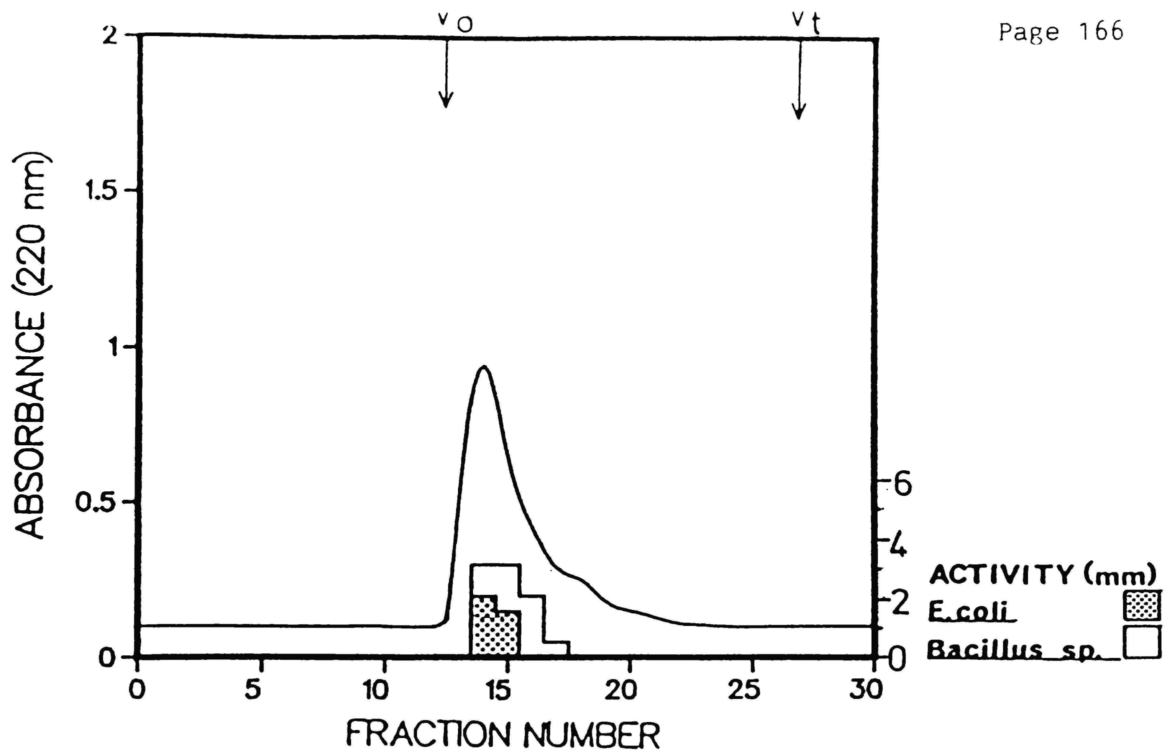


Fig. 6-22a: Gel Filtration Chromatography of Fractions 18-28 from Fig. 6-21
Gel: Sephadex G 25 SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 18-28 (Fig. 6-21), salt and urea removed, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

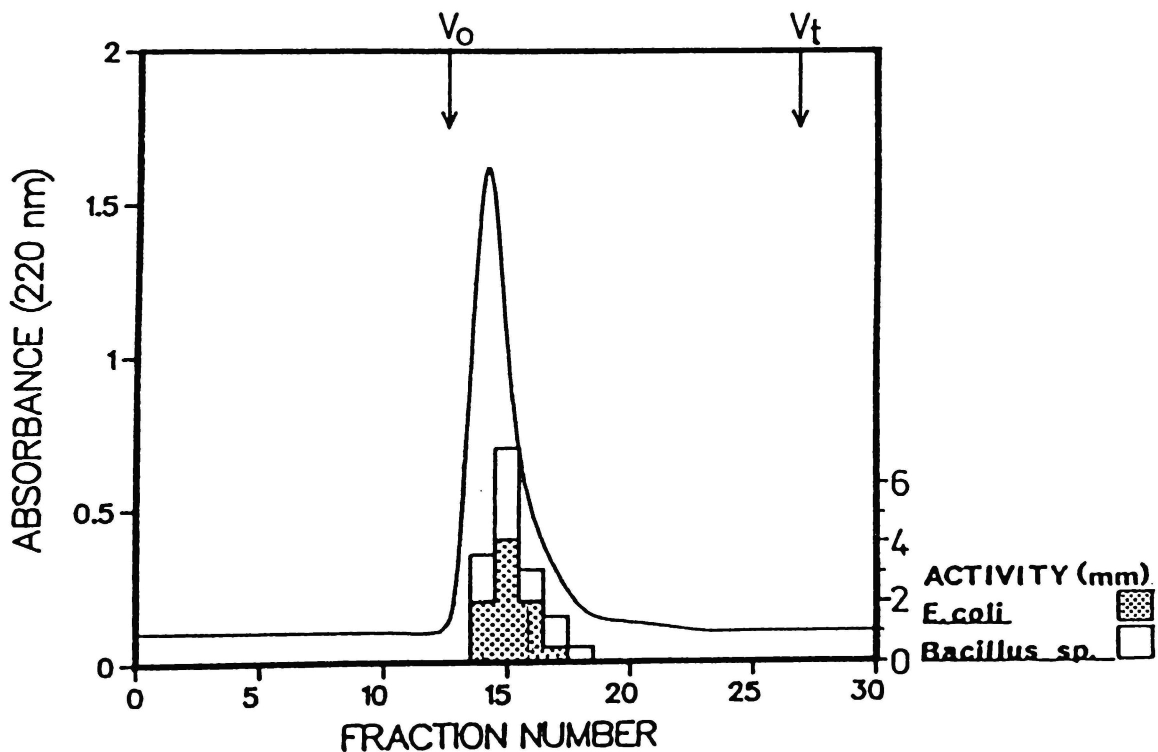


Fig. 6-22b: Gel Filtration Chromatography of Fractions 29-42 from Fig. 6-21
Gel:Sephadex G 25 SF
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 29-42 (Fig. 6-21), salt and urea removed, evaporated to 5ml and adjusted to pH 1.7
Fraction Volume: 10 ml

Plate 6-5: Cationic Electrophoresis

1 - not relevant

2 - lysozyme standard

3 - fraction 14 from Fig. 6-21

4 - fraction 14 from Fig. 6-21

5 - fraction 18 from Fig. 6-21

6 - fraction 18 from Fig. 6-21

7 - fraction 14 from Fig. 6-22

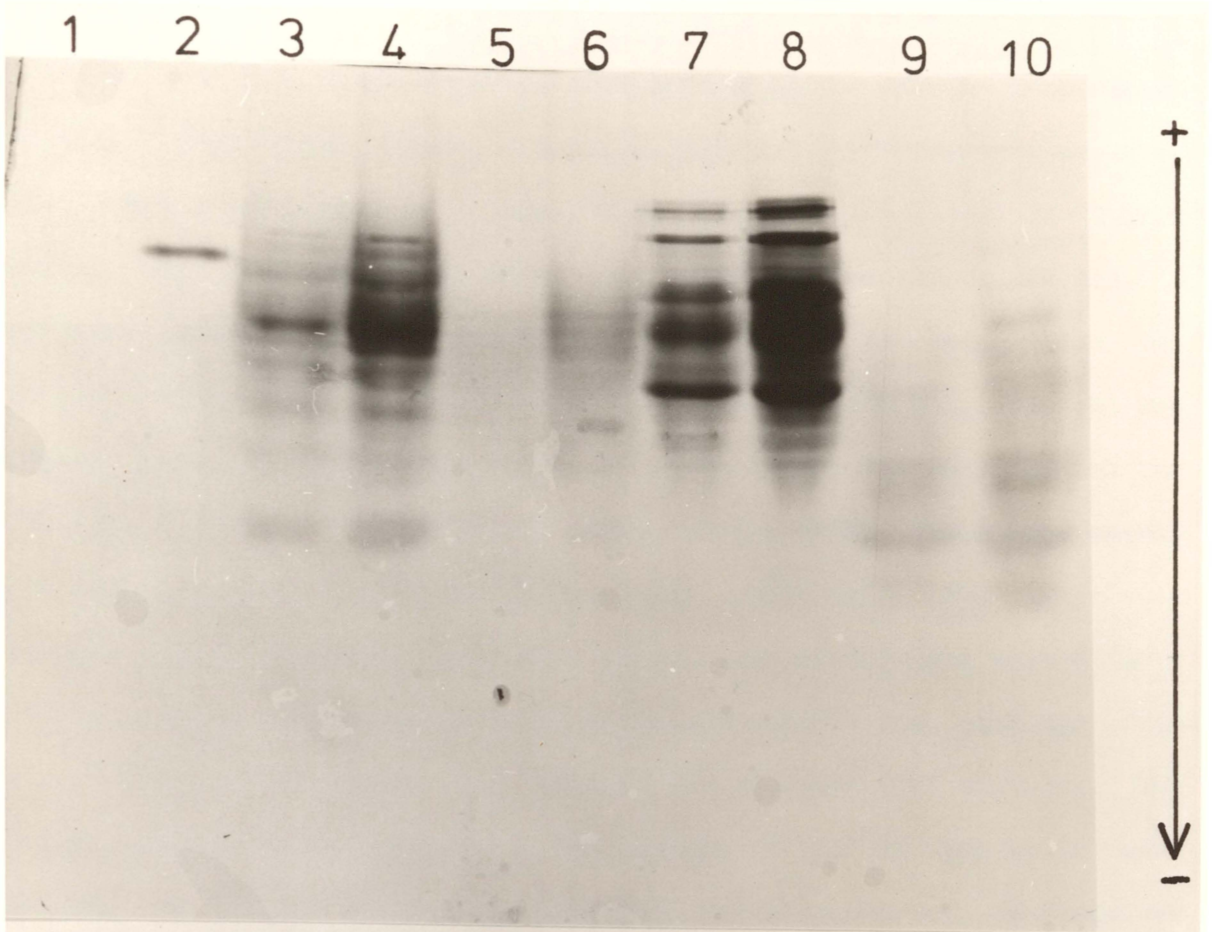
8 - fraction 14 from Fig. 6-22

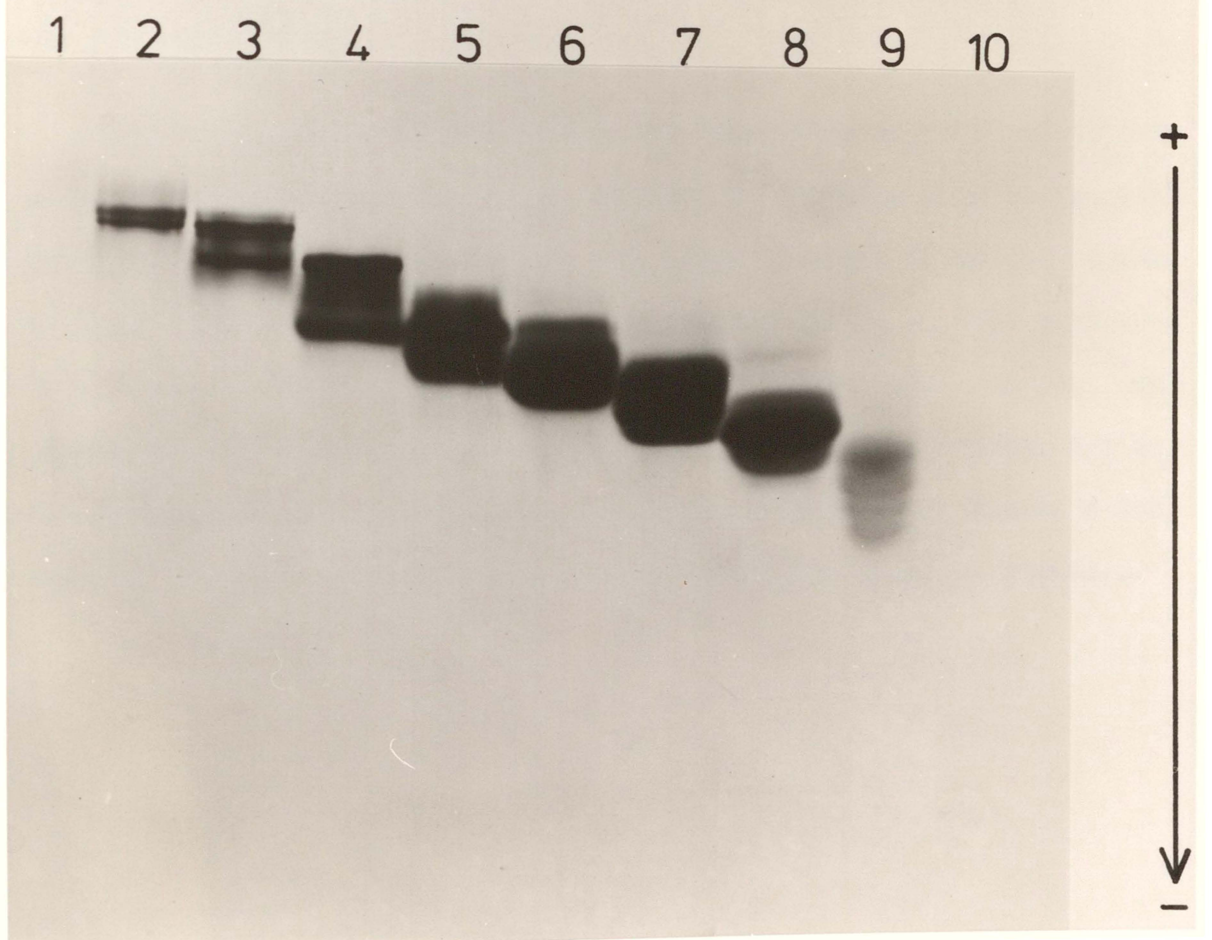
9 - fraction 18 from Fig. 6-22

10- fraction 18 from Fig. 6-22

Plate 6-6: Cationic Electrophoresis

1 - strip 2 from preparative electrophoresis shown in Fig. 6-23
2 - strip 3 from preparative electrophoresis shown in Fig. 6-23
3 - strip 4 from preparative electrophoresis shown in Fig. 6-23
4 - strip 5 from preparative electrophoresis shown in Fig. 6-23
5 - strip 6 from preparative electrophoresis shown in Fig. 6-23
6 - strip 7 from preparative electrophoresis shown in Fig. 6-23
7 - strip 8 from preparative electrophoresis shown in Fig. 6-23
8 - strip 9 from preparative electrophoresis shown in Fig. 6-23
9 - strip 10 from preparative electrophoresis shown in Fig. 6-23
10- strip 11 from preparative electrophoresis shown in Fig. 6-23


1
2
3
4
5
6
7
8
9
10
+
−


1
2
3
4
5
6
7
8
9
10
+
−

Cationic electrophoresis was used as the basis for a preparative method. The major active fractions from the chromatography shown in Fig. 6-22b (fractions 14-16) were freeze-dried, re-dissolved in sample buffer and applied in a wide well formed in the top of the gel. A constant current of 40 mA was applied for 3 hours, in which time the solvent front had migrated almost to the end of the gel. The gel was removed from the chamber and after the glass plates had been removed the gel was cut into 1 cm strips as shown in Fig. 6-23. These were mashed and each soaked in 2 ml of distilled water overnight. The water was drained and collected, and the mashed gel strips were soaked for a further 4 hours in another 2 ml of distilled water. The washings from each strip were pooled and assayed for antibacterial activity. The results are shown in Table 6-1. Gel strips 4 to 10 inclusive were active. A 200 µl sample from each pool was freeze-dried, re-dissolved in sample buffer and run on cationic electrophoresis. Separation of some bands was achieved (see Plate 6-6), but each active pool had more than one band present.

The washings from gel strips 4 to 10 (approximately 4 ml each) were separately chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl pH 1.7. The results are shown in Figs. 6-24a to 6-24g. Fractions from each gel filtration run as marked in Fig. 6-24 were freeze-dried, re-dissolved in SDS sample buffer and run on SDS electrophoresis. An interesting pattern emerged (see Plates 6-7 and 6-8), from which the bands were grouped as shown in Figure 6-24h, according to their migration rate compared with the lysozyme standard.

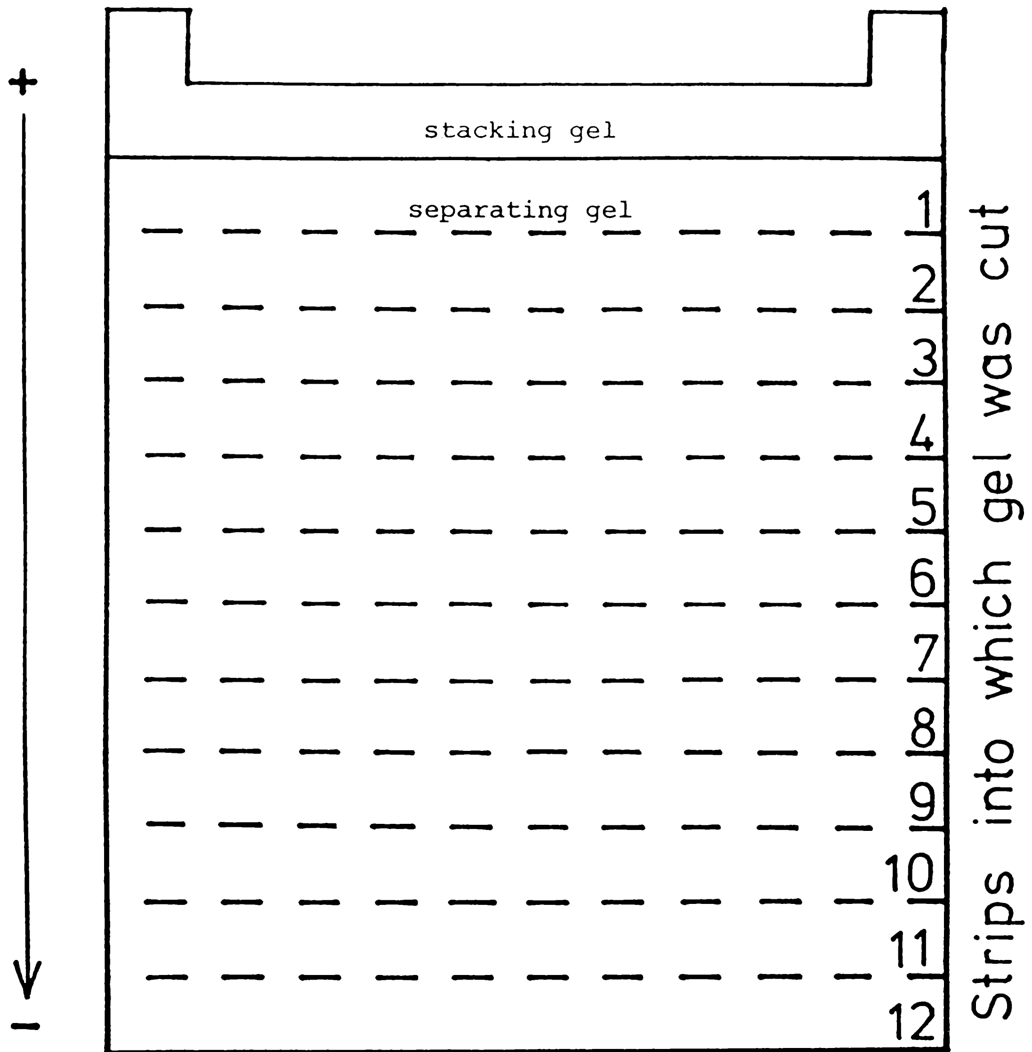


Fig. 6-23: Cutting of Preparative Cationic Electrophoresis Gel

Table 6-1: Antibacterial Activity of Gel Strips 1 to 12 shown in Fig. 6-23

strips	Antibacterial Activity (mm)	
	Bacillus sp.	E. coli
1	0	0
2	0	0
3	0	0
4	1.5	1
5	3	2
6	5	3
7	6	3
8	5	3
9	7	3
10	4	2
11	0	0
12	0	0

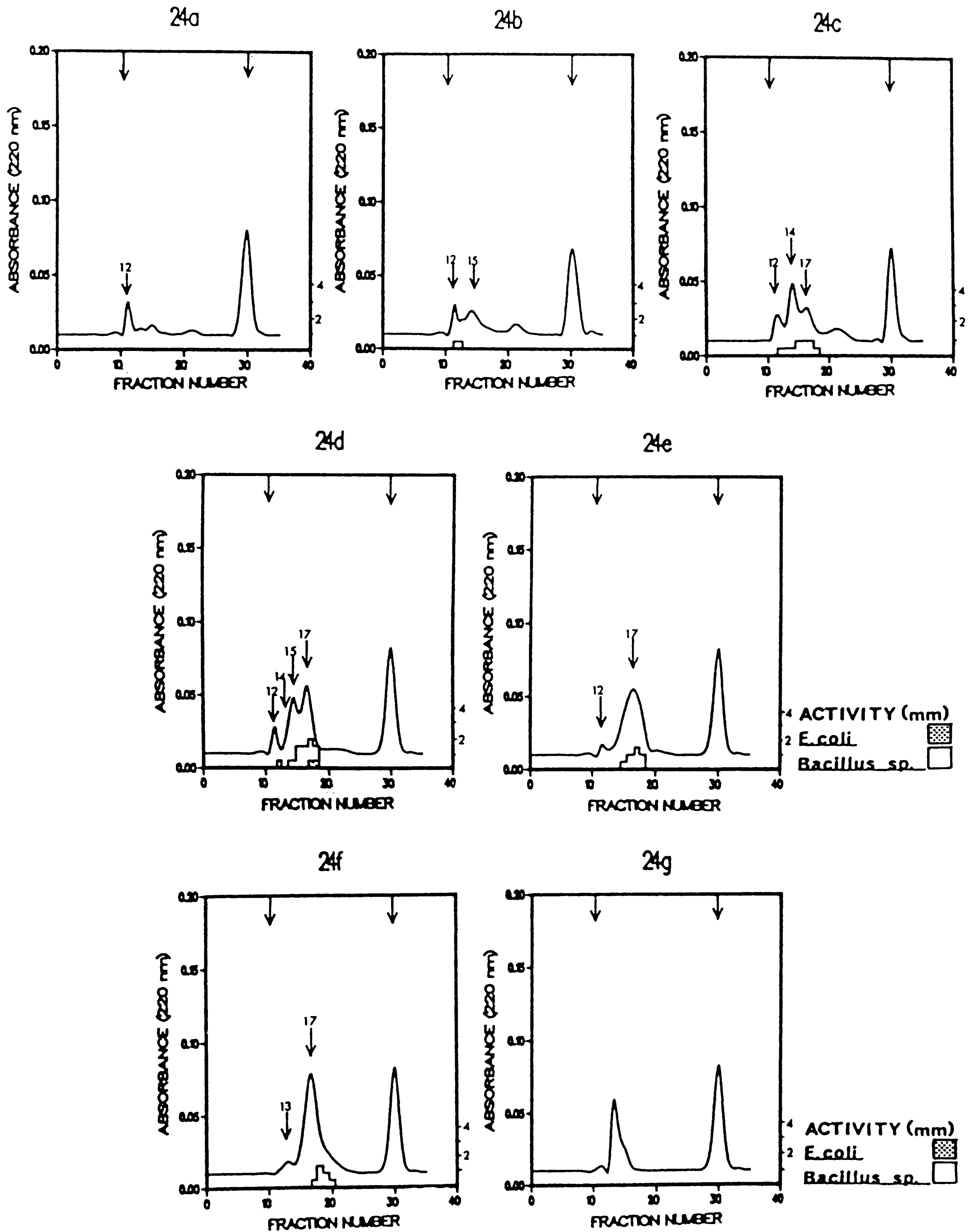


Fig. 6-24a-g: Gel Filtration Chromatography of Gel Strips 4-10 from Fig. 6-23
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Gel strip solutions 4 to 10 from preparative electrophoresis shown in Fig. 6-23, adjusted to pH 1.7
Fraction Volume: 10 ml

Plate 6-7: SDS Electrophoresis

1 - lysozyme standard

2 - fraction 12 from Fig. 6-24a

3 - fraction 12 from Fig. 6-24b

4 - fraction 14 from Fig. 6-24b

5 - fraction 12 from Fig. 6-24c

6 - fraction 14 from Fig. 6-24c

7 - fraction 17 from Fig. 6-24c

8 - not relevant

9 - not relevant

10- lysozyme standard

Plate 6-8: SDS Electrophoresis

1 - lysozyme standard

2 - fraction 17 from Fig. 24f

3 - fraction 13 from Fig. 24f

4 - fraction 17 from Fig. 24e

5 - fraction 12 from Fig. 24e

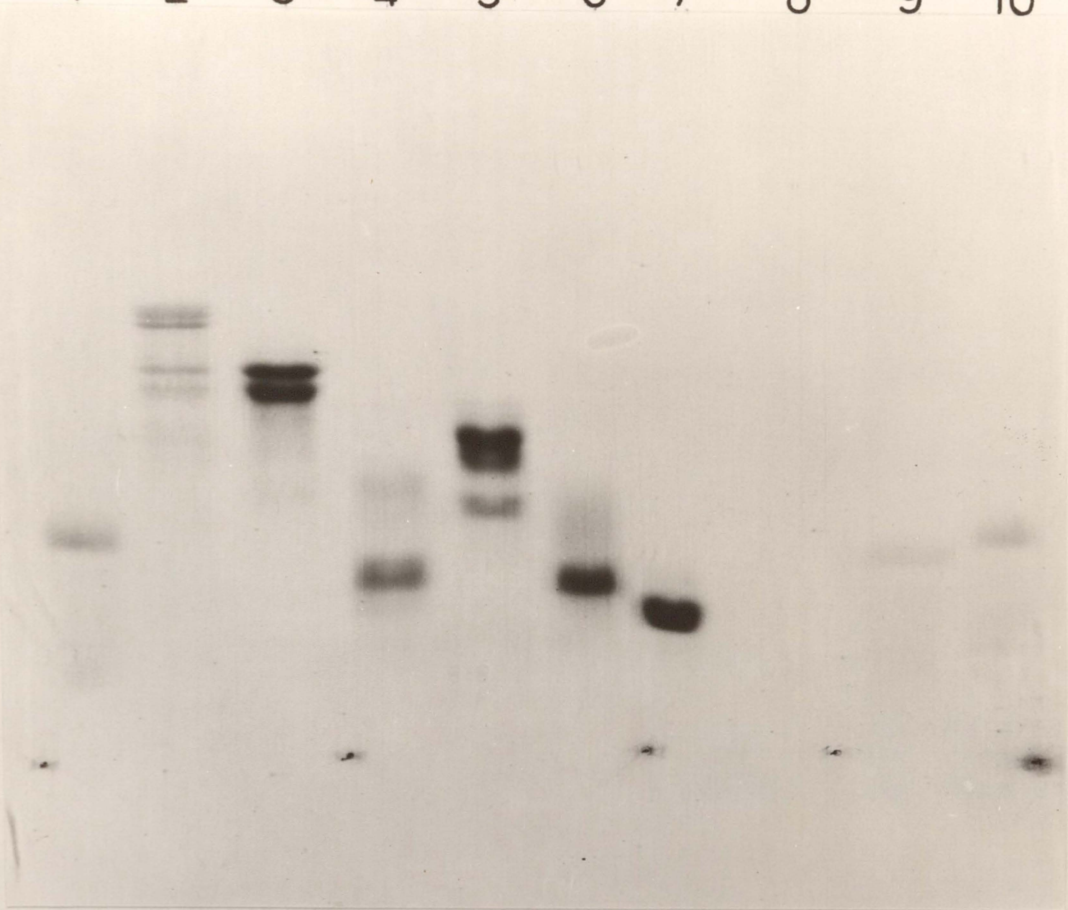
6 - fraction 17 from Fig. 24d

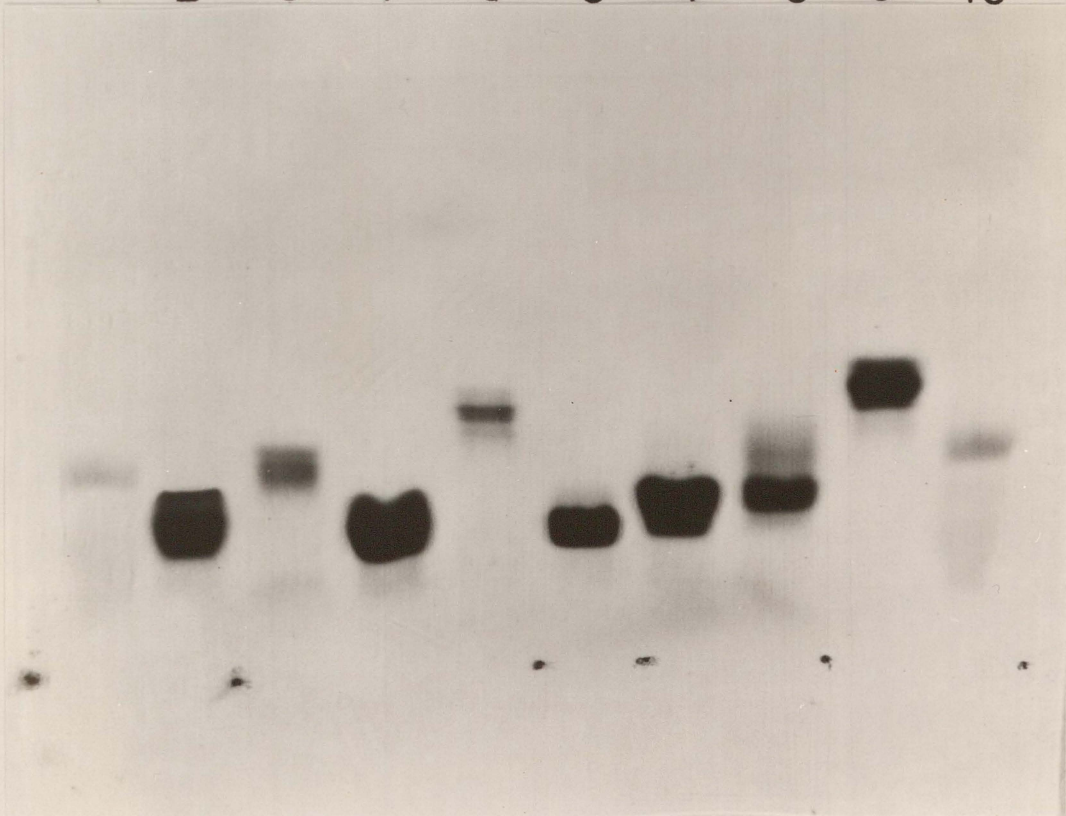
7 - fraction 15 from Fig. 24d

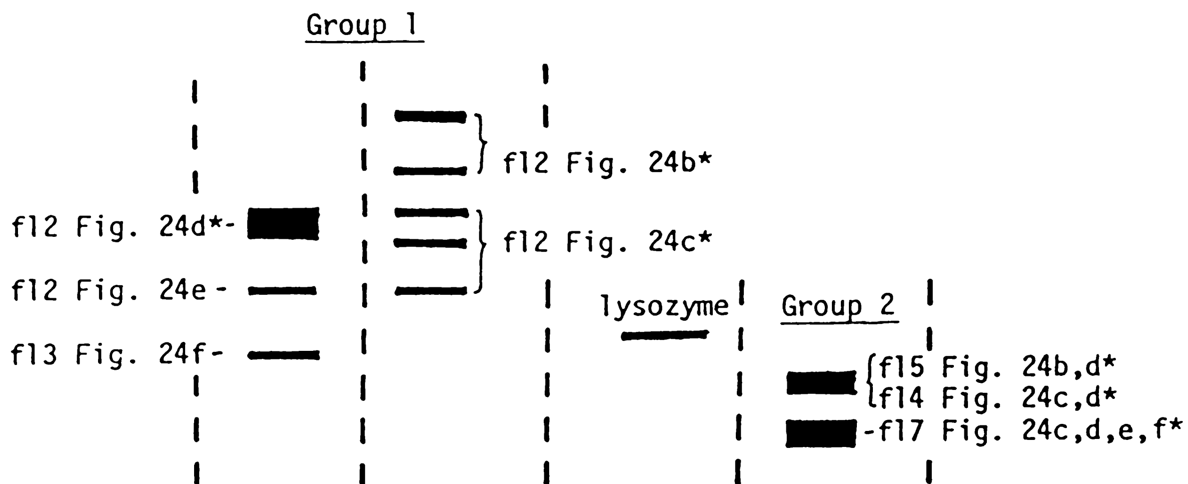
8 - fraction 14 from Fig. 24d

9 - fraction 12 from Fig. 24d

10- lysozyme standard


1 2 3 4 5 6 7 8 9 10
−
+


1 2 3 4 5 6 7 8 9 10
−
+



*antibacterial activity in fraction before electrophoresis

<u>Fig. 6-24h</u>: Comparison of the migration rates of the bands shown in Plates 6-7 and 6-8 and the lysozyme standard

Group 1 had a migration rate slower than lysozyme. It consisted of several bands of which at least two must have been antibacterial. Fraction 12 (Fig. 6-24b) contained two bands, at least one of which must have been responsible for the antibacterial activity. Fraction 12 (Fig. 6-24c) contained 3 bands of which at least one must have been responsible for the antibacterial activity. All of these bands are different from those from fraction 12 (Fig. 6-24b). Fraction 12 (Fig. 6-24d) contained one broad band which had a similar mobility to that from fraction 12 (Fig. 6-24c). Fraction 12 (Fig. 6-24e) and fraction 13 (Fig. 6-24f) were not active. Therefore there are at least two active components in Group 1.

Group 2 had a migration rate faster than lysozyme. It consisted of two bands, both which must have antibacterial activity. The first band, of higher molecular weight, was present in fraction 15 (Fig. 6-24b), fraction 15 (Fig. 6-24d), fraction 14 (Fig. 6-24c) and fraction 14 (Figs. 6-24d). The second band, of lower molecular weight, was present in fractions 17 (Fig. 6-24c), fraction 17 (Fig. 6-24d), fraction 17 (Fig. 6-24e) and fraction 17 (Fig. 6-24f). The bands in fraction 17 (Fig. 6-24e) and fraction 17 (Fig. 6-24f) were very broad and may represent more than one component.

The same fractions were also run on cationic electrophoresis. The results (shown in Plates 6-9 and 6-10) further support the groupings made from the results of the SDS electrophoresis. In Group 1 fractions 12 (Figs. 6-24b) and and fraction 12 (Fig. 6-24c) have different mobilities and hence appear to be two different compounds of high molecular weight. In Group 2 fractions 15 (Fig. 6-24b) and 14 (Fig. 6-24c) appear to consist of a band with the same mobility as do fractions 14 and 15 (Fig. 6-24d). However it is difficult to compare

Plate 6-9: Cationic Electrophoresis

1 - fraction 28 from Fig. 24e

2 - fraction 17 from Fig. 24e

3 - fraction 12 from Fig. 24e

4 - fraction 22 from Fig. 24c

5 - fraction 14 from Fig. 24c

6 - fraction 17 from Fig. 24c

7 - fraction 12 from Fig. 24c

8 - fraction 22 from Fig. 24b

9 - fraction 15 from Fig. 24b

10- fraction 12 from Fig. 24b

Plate 6-10: Cationic Electrophoresis

1 - lysozyme standard

2 - fraction 32 from Fig. 24g

3 - fraction 30 from Fig. 24g

4 - fraction 14 from Fig. 24g

5 - fraction 17 from Fig. 24f

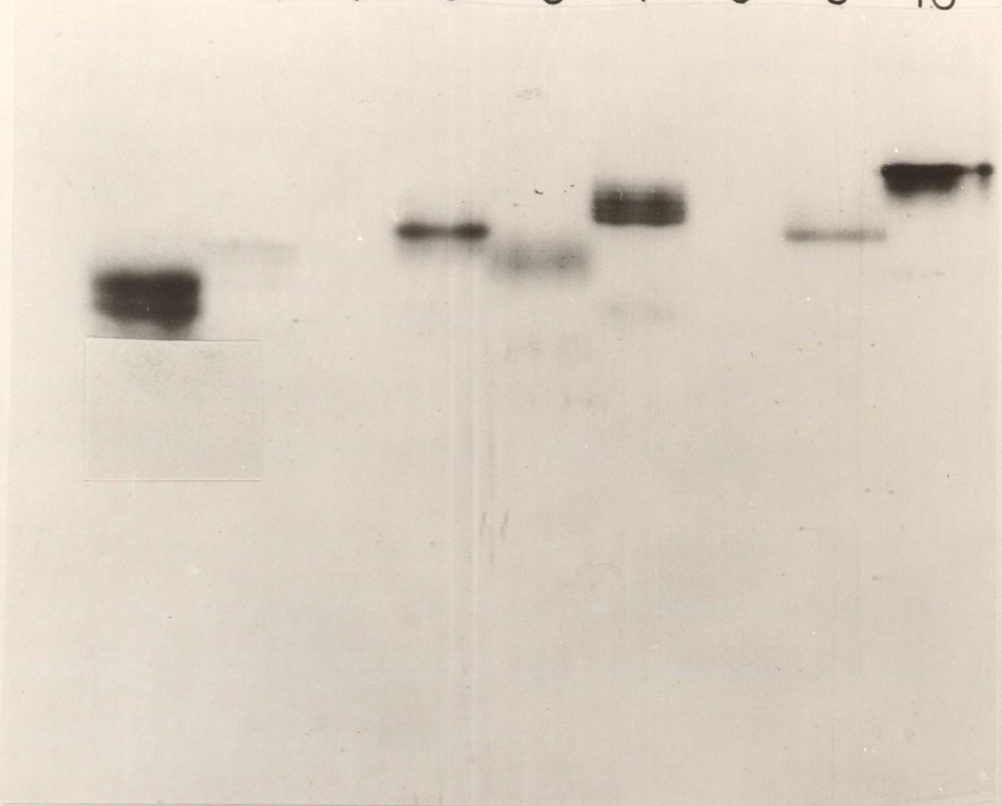
6 - fraction 13 from Fig. 24f

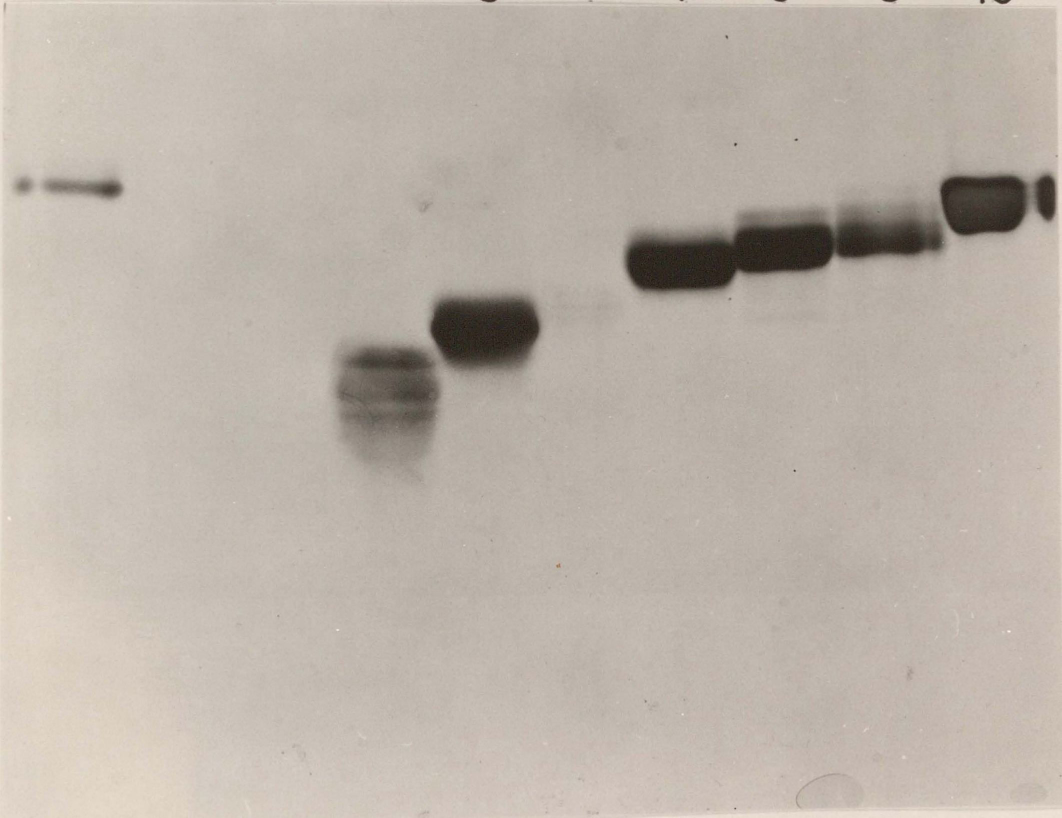
7 - fraction 17 from Fig. 24d

8 - fraction 15 from Fig. 24d

9 - fraction 14 from Fig. 24d

10- fraction 12 from Fig. 24d


1 2 3 4 5 6 7 8 9 10
+
−


1 2 3 4 5 6 7 8 9 10
+
−

the two gels as there were no reference standards or R_f values to use. Even so, the indication was that there was one band present in this group. The component of low molecular weight does appear to consist of more than one band. Fraction 17 (Fig. 6-24d) and fraction 17 (Fig. 6-24f) contain bands with quite different mobilities. Fraction 17 (Fig. 6-24c) also has a different mobility from fraction 17 (Fig. 6-24e) which has two distinct bands. Thus there appears to be at least two different active components of low molecular weight.

The active fractions shown in Fig. 6-22a (fractions 14-16) were also run on preparative cationic electrophoresis and the bands were investigated in the same way. Similar results were obtained with these fractions but with a smaller yield.

Conclusions

These results suggest that there are at least five different antibacterial components present in the sheep thymus preparation. However the major disadvantage of preparative electrophoresis is the small amount of protein that can be separated from each run. Also it was necessary for further purification on gel filtration chromatography before the samples were at the stage presented here. The quantity of material was only just sufficient for adequate detection using the well diffusion assay method. Therefore alternative separation methods were used in an attempt to isolate the antibacterial compounds in larger quantities.

6.6 Isolation of the Sheep Thymus Antibacterial Proteins using EDTA

EDTA was used successfully in helping to dissociate the antibacterial proteins in the milk cell extract. Furthermore, Molan (personal communication), found that dialysis of samples in the presence of 0.1 mol/l EDTA helped in the dissociation of cationic proteins in bovine seminal plasma.

The sheep thymus preparation of Dubos and Hirsch (1954) was first chromatographed through a column of Sephadex G 25 eluted with 0.02 mol/l HCl to remove the polyamines present. The active fractions eluted from the void volume of the column were separated from the polyamines and freeze-dried. The freeze-dried powder (100 mg) was re-dissolved in 10 ml of 0.1 mol/l EDTA (sodium salt, pH 7). The thymus peptide was dialysed against 3 changes of 20 ml of 0.1 mol/l EDTA (sodium salt, pH 7) for 72 hours at room temperature with the diffusate being collected every 24 hours and stored. The dialysis tubing used had a nominal molecular weight cut-off at 3 500. The retentate was adjusted to pH 1 by the addition of concentrated HCl. A heavy precipitate formed which was primarily the EDTA falling out of solution. The precipitate was removed by centrifugation and the remaining solution was adjusted to pH 1.7 and chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl (pH 1.7). A very broad elution profile resulted with antibacterial activity spread throughout (see Fig. 6-25). Comparing Fig. 6-25 with Fig. 6-8 it appears that the EDTA dialysis may have caused more aggregation rather than disaggregation. However, during the purification of the milk cell extract the gel filtration chromatography in the presence of EDTA did not appear to cause disaggregation (as it gave a very broad elution profile), yet it facilitated the isolation of the antibacterial compounds.

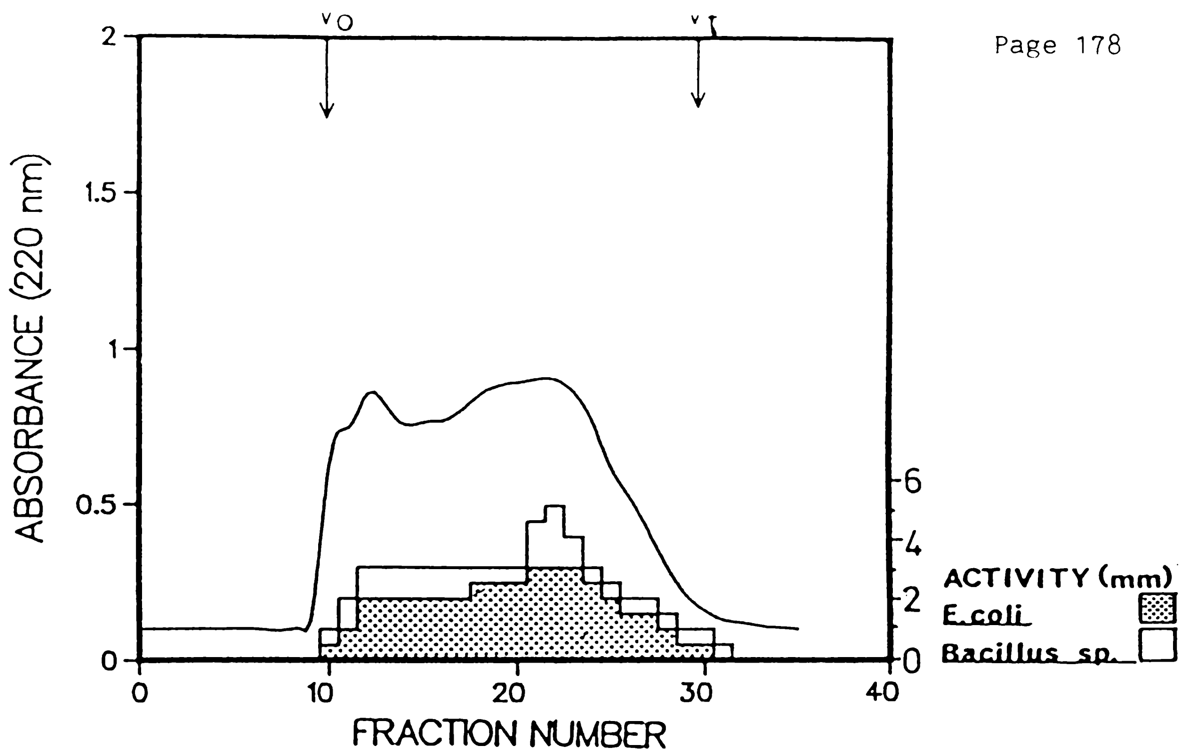


Fig. 6-25: Gel Filtration Chromatography of the Retentate from EDTA Dialysis of the Sheep Thymus Preparation
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Retentate from EDTA dialysis of the sheep thymus preparation, adjusted to pH 1, precipitate removed and re-adjusted to pH 1.7
Fraction Volume: 10 ml

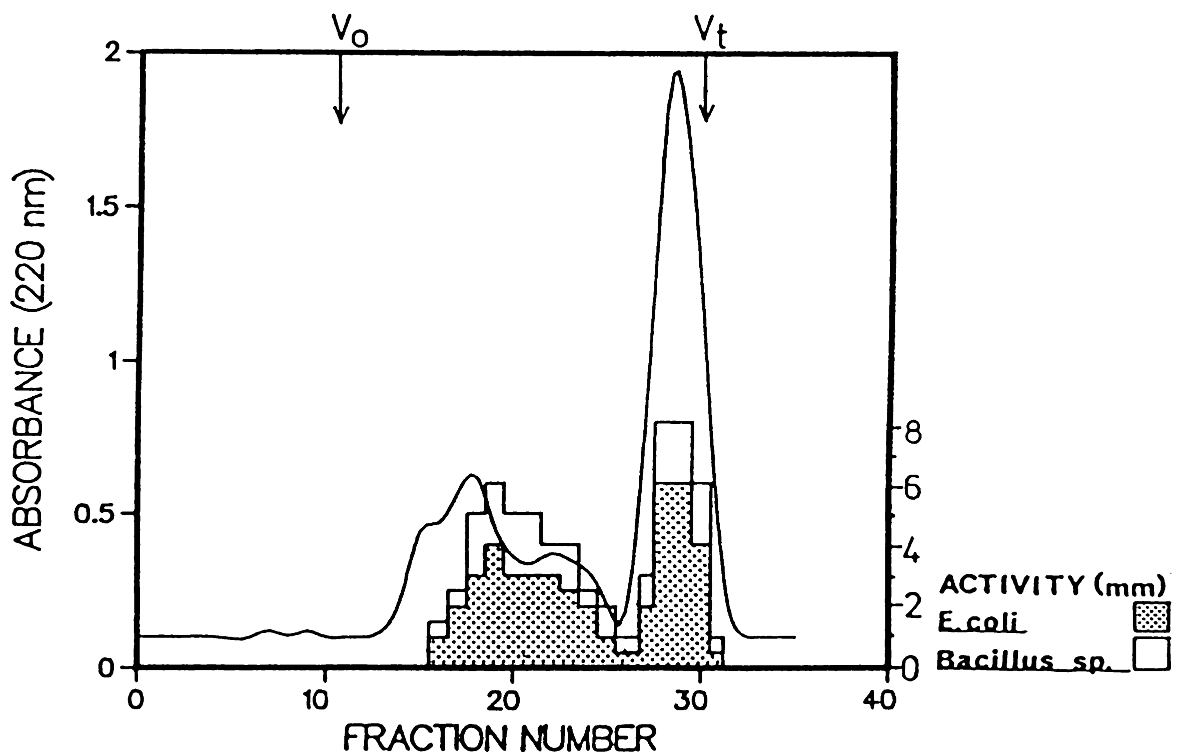


Fig. 6-26: Gel Filtration Chromatography of the Re-dissolved Precipitate
Gel: Sephadex G 50 SF
Eluent: Distilled water
Sample: Precipitate removed from the retentate at pH 1, re-dissolved in 10 ml of distilled water and adjusted to pH 7
Fraction Volume: 10 ml

Samples of 0.5 ml were taken from every alternate fraction from 11 to 27, freeze-dried, re-dissolved in SDS sample buffer and examined on SDS electrophoresis for evidence of separation. The results are shown in Plate 6-11. The electrophoretic pattern showed some evidence of separation between high molecular weight and low molecular weight regions of the elution profile, but there still remained a number of bands in each sample. With consideration of the separation that was achieved the fractions were divided and pooled as follows: fractions 11-14, 15-18 and 19-30.

The precipitate that formed when adjusting the retentate from EDTA dialysis to pH 1 was re-dissolved by returning the pH to 7 with NaOH, and was run through a column of Sephadex G 50 eluted with distilled water. A broad active peak was eluted (see Fig. 6-26), followed by a large peak of EDTA at the bed volume (EDTA is antibacterial). Samples of 0.5 ml were taken from alternate fractions from 16 to 24, freeze-dried, and re-dissolved in SDS sample buffer. These fractions were examined on SDS electrophoresis as shown in Plate 6-12. Fractions 16 and 18 contained many bands of high molecular weight but fractions 20, 22 and 24 were predominantly of low molecular weight.

Similar procedures were also carried out with the diffusate from the EDTA dialysis of the sheep thymus preparation, to find if any antibacterial peptides had passed through the membrane. Some peptides of low molecular weight were present but did not exhibit any antibacterial activity and were therefore discarded.

Further purification of the dialysed antibacterial compounds was attempted. Fractions 19-31 (Fig. 6-25) were chosen first as they appeared to be of predominantly lower molecular weight and less complex

Plate 6-11: SDS Electrophoresis

1 - fraction 11 from Fig. 6-25

2 - fraction 13 from Fig. 6-25

3 - fraction 15 from Fig. 6-25

4 - fraction 17 from Fig. 6-25

5 - fraction 19 from Fig. 6-25

6 - fraction 21 from Fig. 6-25

7 - fraction 23 from Fig. 6-25

8 - fraction 25 from Fig. 6-25

9 - fraction 27 from Fig. 6-25

10- lysozyme, β-lactoglobulin and trypsinogen standards

Plate 6-12: SDS Electrophoresis

1 - lysozyme, β-lactoglobulin and trypsinogen standards

2 - not relevant

3 - not relevant

4 - not relevant

5 - not relevant

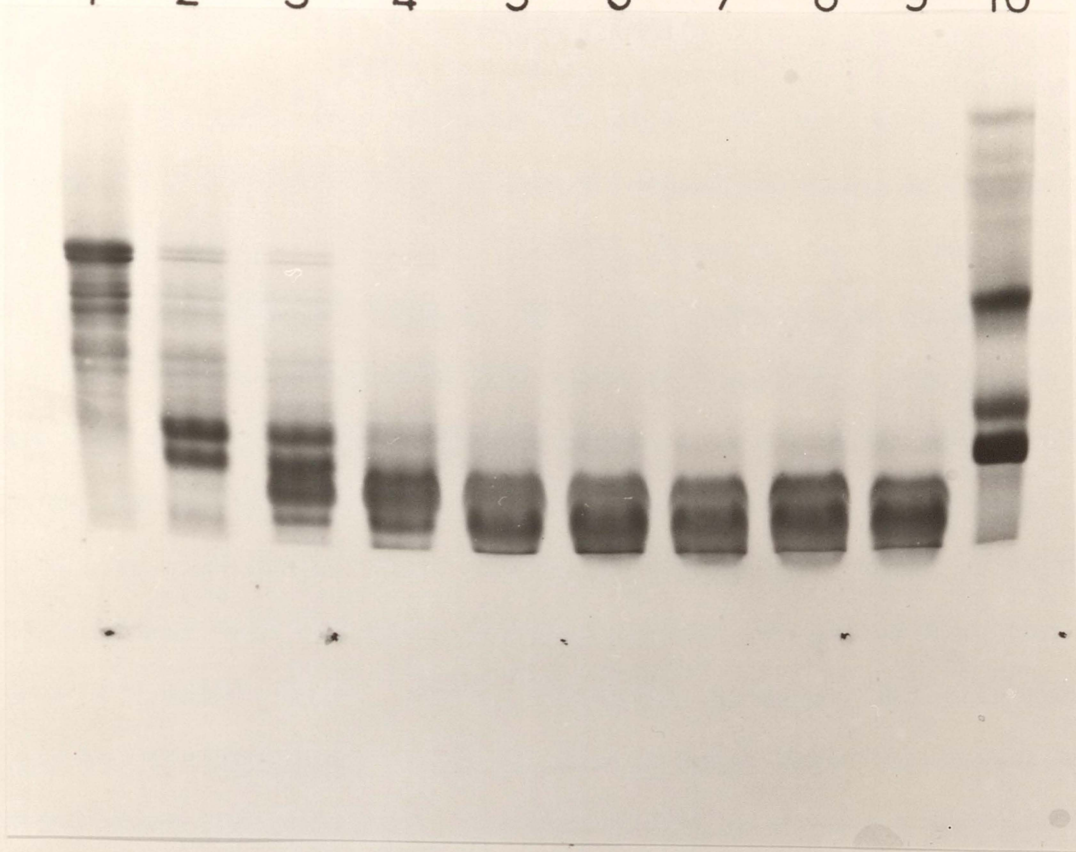
6 - fraction 24 from Fig. 6-26

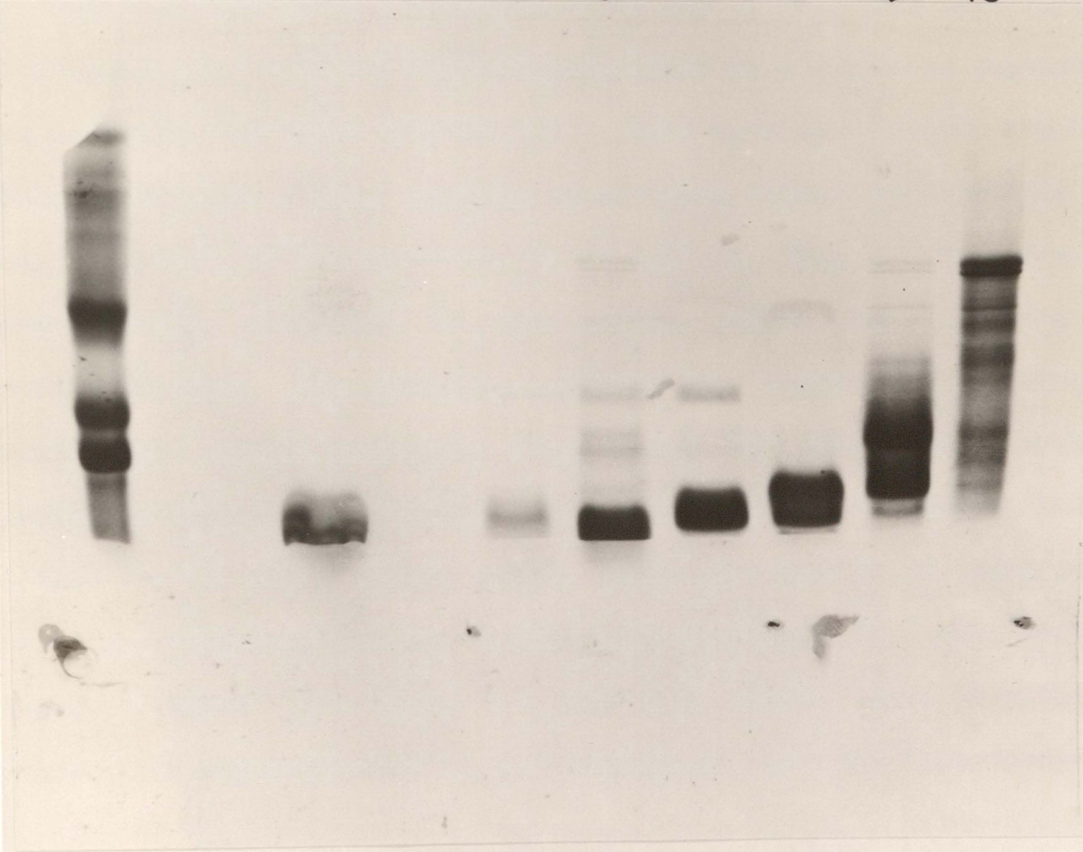
7 - fraction 22 from Fig. 6-26

8 - fraction 20 from Fig. 6-26

9 - fraction 18 from Fig. 6-26

10- fraction 16 from Fig. 6-26


1 2 3 4 5 6 7 8 9 10
−
+


1 2 3 4 5 6 7 8 9 10
−
+

than the higher molecular weight fractions.

The fractions were adjusted to pH 7 and run onto a column of CM Sephadex C 25 equilibrated with 0.1 mol/l phosphate buffer pH 7. The column was eluted with a salt gradient from 0 to 1.0 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 7, followed by a final wash with 0.1 mol/l NaOH. The results are shown in Fig. 6-27. The antibacterial activity was eluted from the salt gradient in a similar position to that in earlier ion exchange runs. The active fractions 31-54 were diluted 1:1 with distilled water and re-run through the column of CM Sephadex C 25, washed with 0.1 mol/l phosphate buffer and eluted from the column with 0.1 mol/l NaOH. These fractions displayed activity against E. coli and the Bacillus species as well as lytic activity against pregrown Micrococcus lysodeikticus. The active fractions were evaporated to 5 ml and chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl. The results are shown in Fig. 6-28. The active fractions were divided into two pools, fractions 15-17 and 18-21. They were separately evaporated to 5 ml and re-chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl (pH 1.7). Both runs gave broad peaks (see Figs. 6-29 and 6-30). Samples (1 ml) were taken from fractions 13, 15 and 17 (Fig 6-29) and fraction 16, 18 and 20 (Fig. 6-30). They were freeze-dried, dissolved in SDS sample buffer and separated by SDS electrophoresis. The fractions appeared as single broad bands (see Plate 6-13) which indicated that there may have been more than one protein present in each band. The bands had an estimated molecular weight of 11 000. These broad bands are similar to those found in fraction 15 (Fig. 6-24b and 6-24d) and fraction 14 (Fig. 6-24c and 6-24d), shown in Plates 6-7 and 6-8, which were obtained from preparative electrophoresis. Although these bands were overloaded they

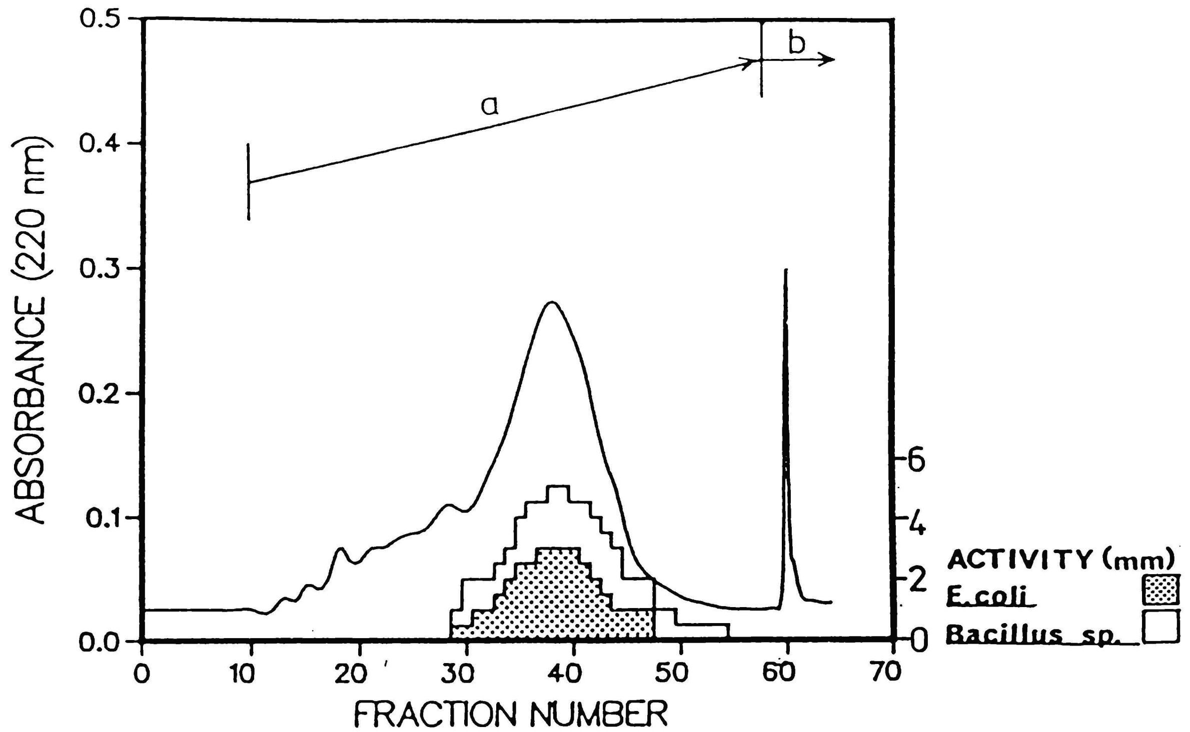


Fig. 6-27: Ion Exchange Chromatography of Fractions 19-31 from Fig. 6-25
Gel: CM Sephadex C 25
Buffer: 0.1 mol/l phosphate, pH 7
Sample: Fractions 19-31 (Fig. 6-25) adjusted to pH 7
Elution Buffers: a- salt gradient from 0 to 1 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 7; b- 0.1 mol/l NaOH
Fraction Volume: 10 ml

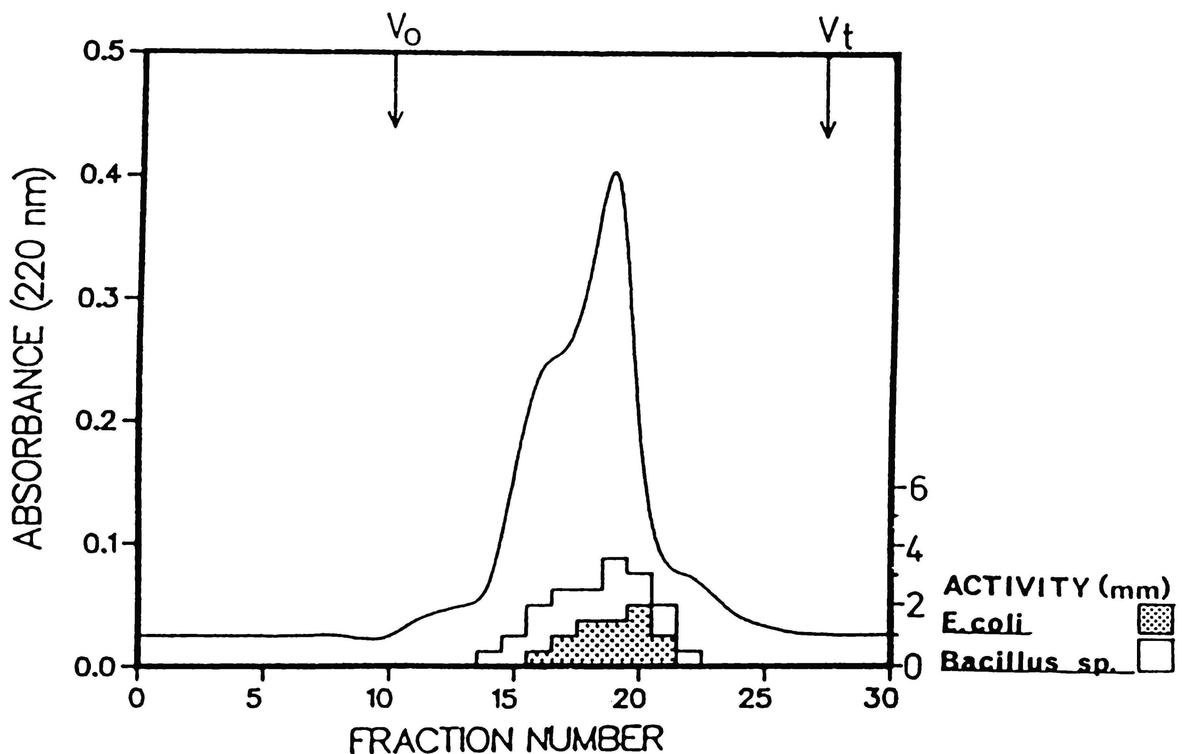


Fig. 6-28: Gel Filtration Chromatography of Fraction 31-54 from Fig. 6-27
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 31-54 (Fig. 6-27), desalted, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

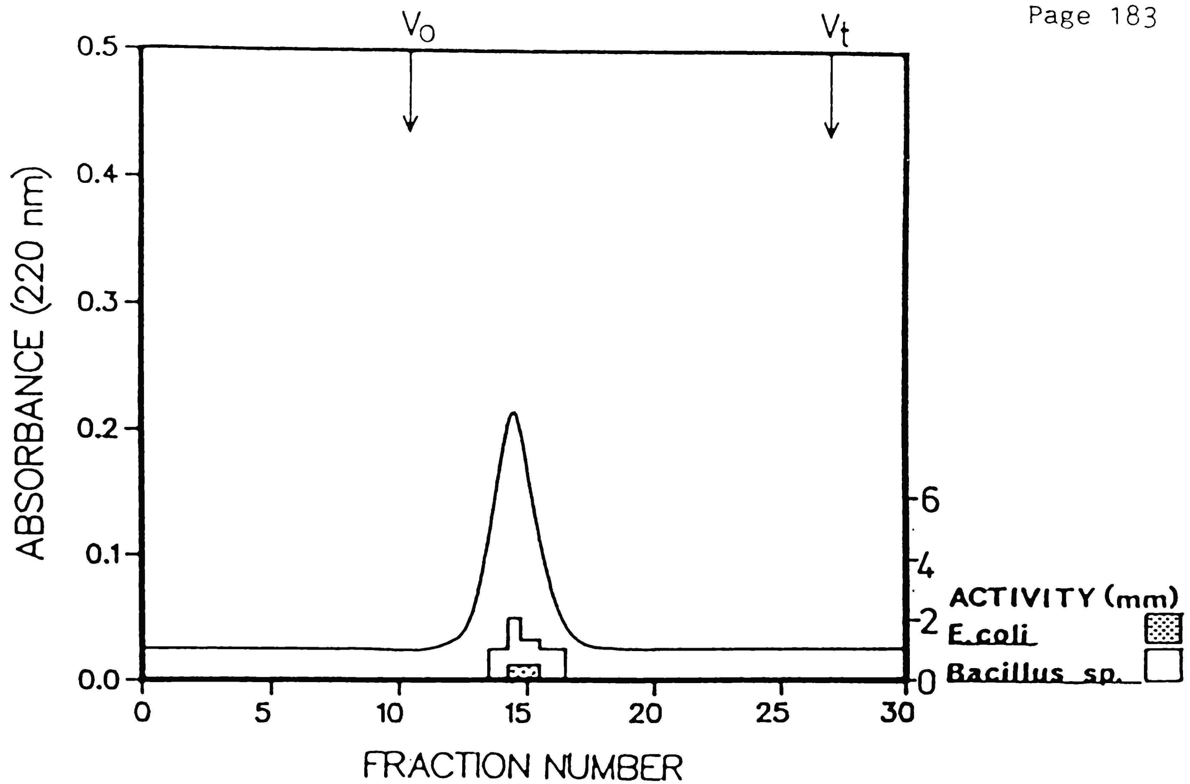


Fig. 6-29: Gel Filtration Chromatography of Fractions 15-17 from Fig. 6-28.
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 15-17 (Fig. 6-28) evaporated to 5 ml
Fraction Volume: 10 ml

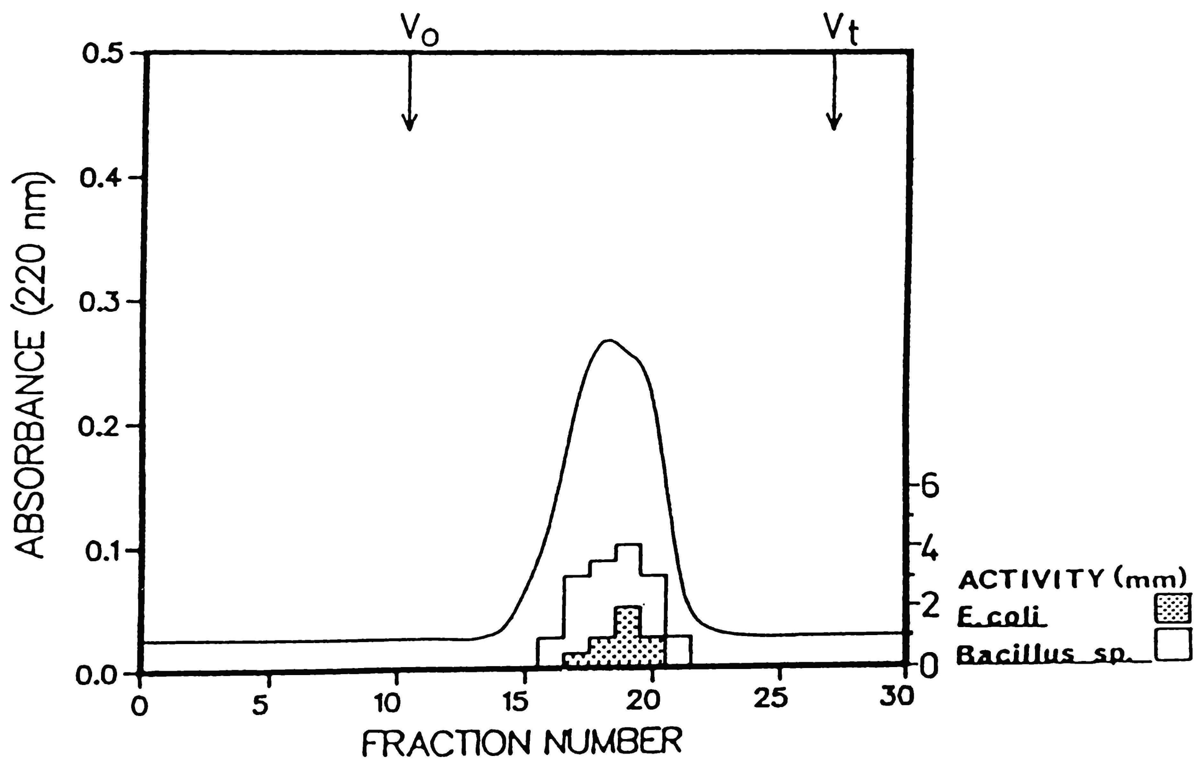


Fig. 6-30: Gel Filtration Chromatography of Fractions 18-21 from Fig. 6-28
Gel:Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 18-21 (Fig. 6-28) evaporated to 5 ml
Fraction Volume: 10 ml

Plate 6-13: SDS Electrophoresis

1 - lysozyme, β-lactoglobulin and trypsinogen standards

2 - not relevant

3 - not relevant

4 - fraction 13 from Fig. 6-29

5 - fraction 15 from Fig. 6-29

6 - fraction 17 from Fig. 6-29

7 - fraction 16 from Fig. 6-30

8 - fraction 18 from Fig. 6-30

9 - fraction 20 from Fig. 6-30

10- α-lactalbumin, trypsin inhibitor, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin standards

Plate 6-14: SDS Electrophoresis

1 - not relevant

2 - not relevant

3 - not relevant

4 - not relevant

5 - fraction 12 from Fig. 6-33

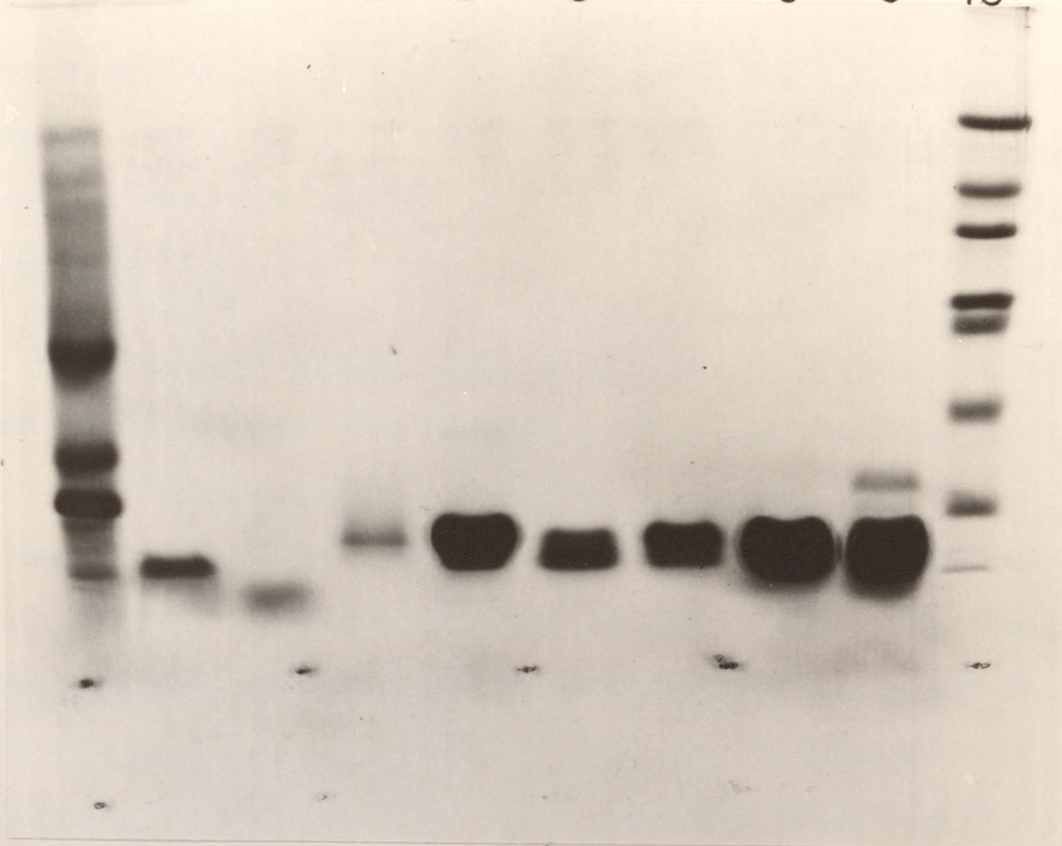
6 - fraction 10 from Fig. 6-33

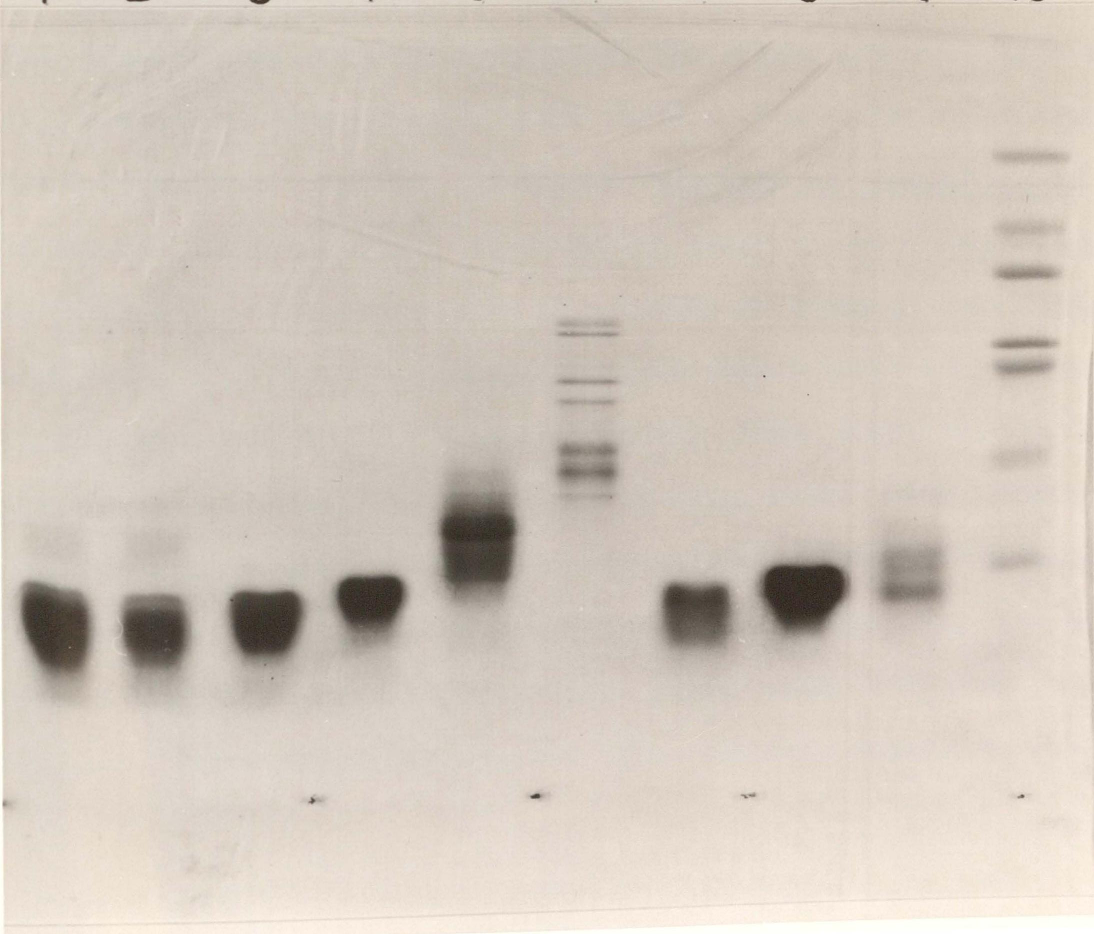
7 - fraction 17 from Fig. 6-34

8 - fraction 15 from Fig. 6-34

9 - fraction 13 from Fig. 6-34

10- α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin standards


1
2
3
4
5
6
7
8
9
10
−
+


1
2
3
4
5
6
7
8
9
10
−
+

were still broad when less protein was added. This could have been a result of the cationic nature of these proteins and their binding with the SDS. Purification of these fractions was left at this stage and further work was carried out on the other active fractions shown in Fig. 6-25.

Fractions 15-18 (Fig. 6-25) were pooled and adjusted to pH 7 and run onto a column of CM Sephadex C 25 ion exchanger equilibrated with 0.1 mol/l phosphate buffer, pH 7. The column was eluted with a salt gradient from 0 to 1.0 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 7,followed by a final wash with 0.1 mol/l NaOH. The results are shown in Fig. 6-31. The active fractions 22-44 were pooled and diluted 1:1 with distilled water and run onto the CM Sephadex C 25 column. Following a wash with 0.1 mol/l phosphate buffer (pH 7) the active fractions were removed from the column with 0.1 mol/l NaOH and neutralised. These active fractions were evaporated to 5 ml and chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl. The results are shown in Fig. 6-32. The active fractions were divided into two pools, fractions 10-13 and 14-17. Fractions 14-17 exhibited lytic activity against pre-grown Micrococcus lysodeikticus cells (fractions 10-13 did not). These pooled fractions were each evaporated to 5 ml and re-chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl. Both chromatographs gave single broad active peaks (see Figs. 6-33 and 6-34). Samples (1 ml) from fractions 10 and 12 (Fig. 6-33) and fractions 13, 15 and 17 (Fig. 6-34) were freeze-dried, re-dissolved in SDS sample buffer and separated by SDS electrophoresis. The results are shown in Plate 6-14. Fractions 13, 15 and 17 (Fig. 6-34) gave bands similar to those from fractions shown in Fig 6-29. These fractions also had lytic activity suggesting the

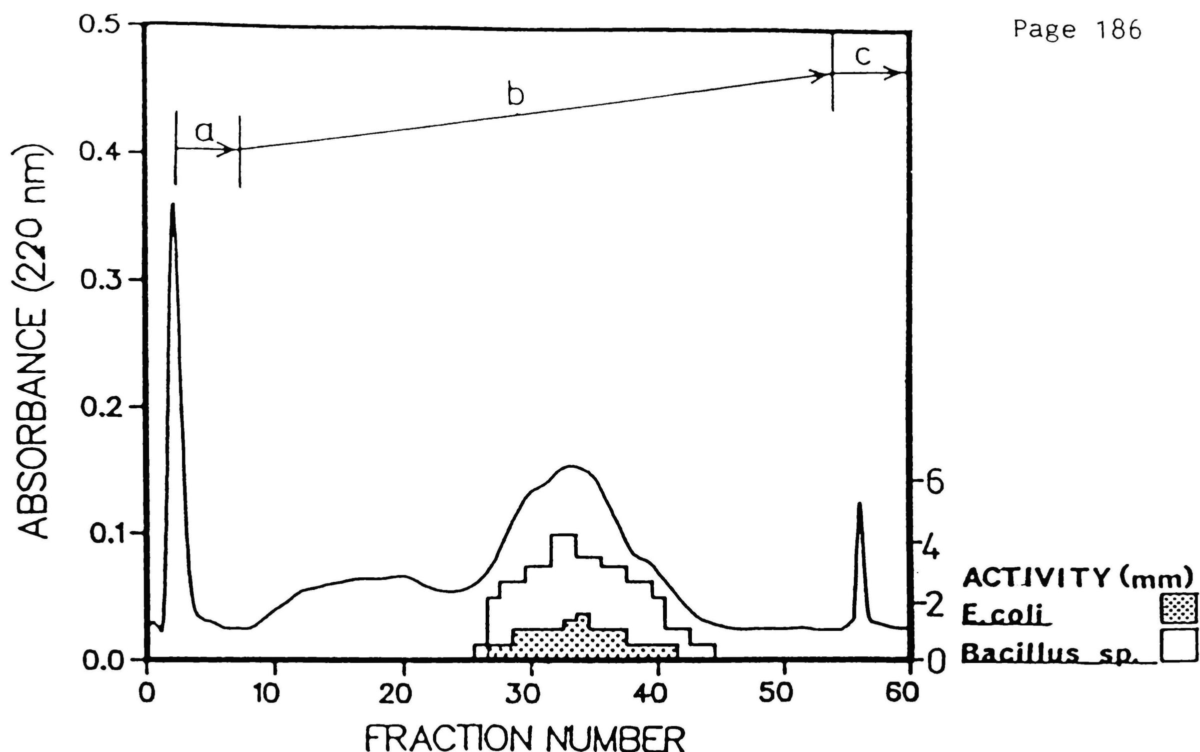


Fig. 6-31: Ion Exchange Chromatography of Fractions 15-18 from Fig. 6-25
Gel: CM Sephadex C 25
Buffer: 0.1 mol/l phosphate, pH 7
Sample: Fractions 15-18 (Fig. 6-25) adjusted to pH 7
Elution Buffers: a- 0.1 mol/l phosphate buffer, pH 7; b- salt gradient from 0 to 1 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 1.7; c- 0.1 mol/l NaOH
Fraction Volume: 10 ml

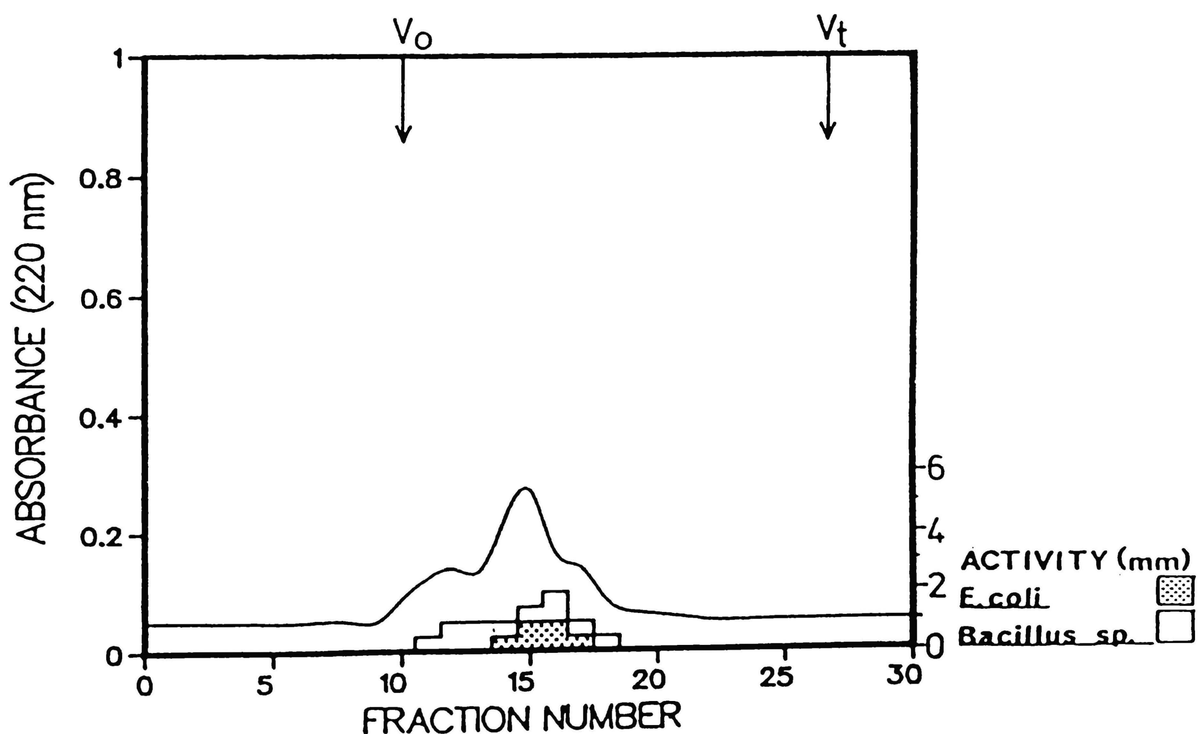


Fig. 6-32: Gel Filtration Chromatography of the Active Fractions from Fig. 6-31
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Active fractions (Fig. 6-31), desalted, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

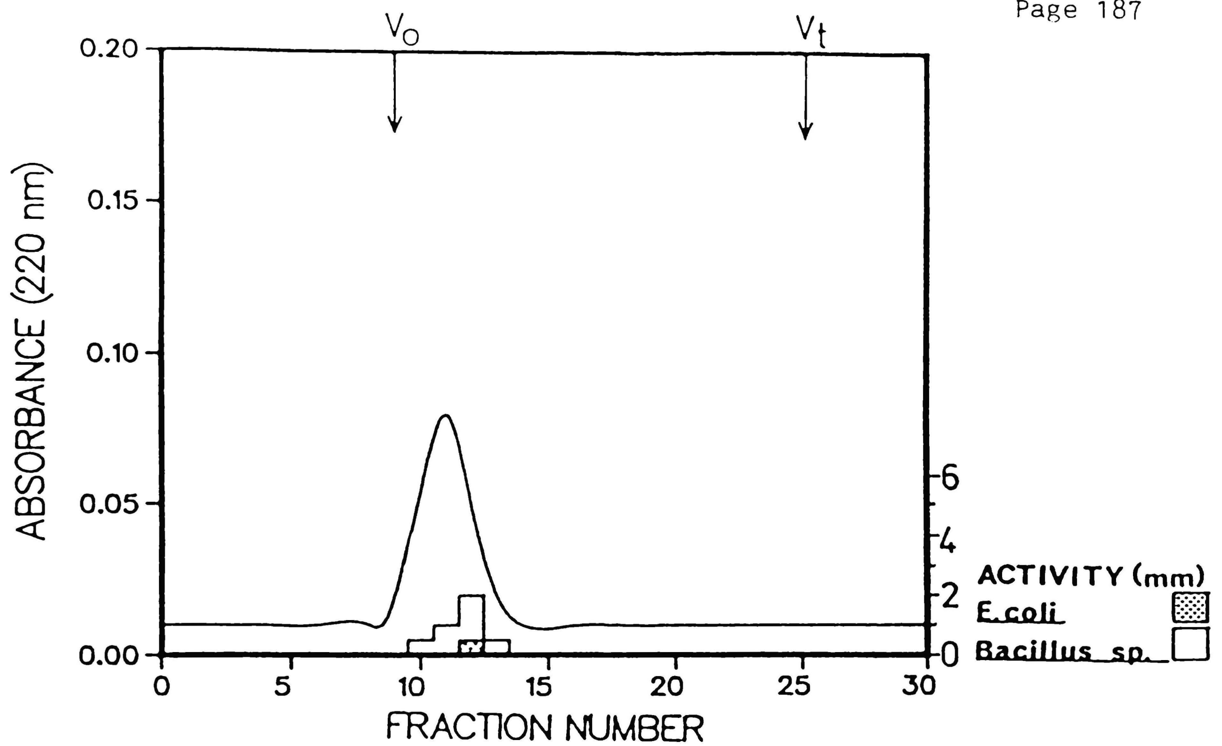


Fig. 6-33: Gel Filtration Chromatography of Fractions 10-13 from Fig. 6-32
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 10-13 (Fig. 6-32) evaporated to 5 ml
Fraction Volume: 10 ml

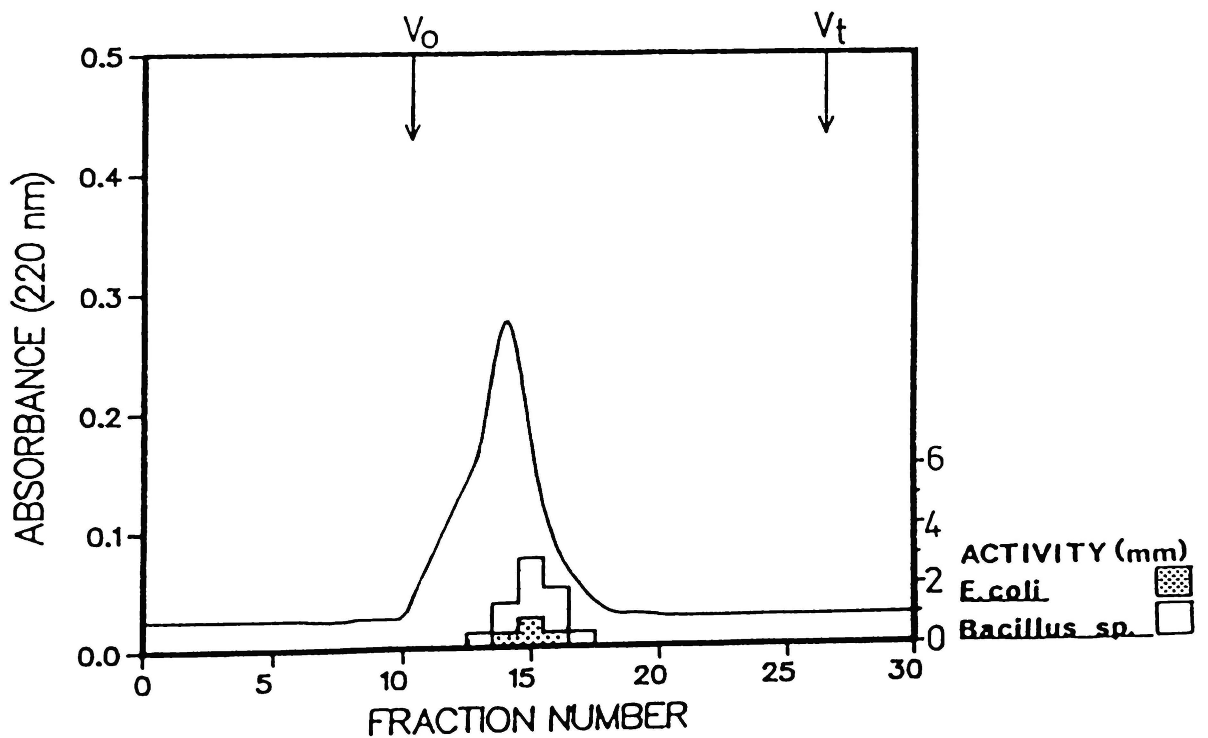


Fig. 6-34: Gel Filtration Chromatography of Fractions 14-17 from Fig. 6-32
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 14-17 (Fig. 6-32) evaporated to 5 ml
Fraction Volume: 10 ml

presence of bovine lysozyme. However these fractions had a faster migration rate than bovine lysozyme (with a molecular weight of 14 000; Eschenbruch, 1980). Therefore it may have been another lytic agent or an unusual aggregate of lysozyme which allowed it to travel further in the gel by binding more SDS hence giving it a higher negative charge. Fraction 10 (Fig. 6-33) showed multiple bands but fraction 12 was predominantly one major band but of higher molecular weight than those from the fractions shown in Fig. 6-34 (molecular weight approximately 16 000)

Finally fractions 11-14 (Fig. 6-25) were adjusted to pH 7 and run onto a column of CM Sephadex C 25 ion exchanger column equilibrated with 0.1 mol/l phosphate buffer pH 7. The column was eluted with a salt gradient from 0 to 1.0 mol/l NaCl in 0.1 mol/l phosphate buffer followed by a final wash with 0.1 mol/l NaOH. The results are shown in Fig. 6-35. The active fractions were eluted as a broad peak by the salt gradient. Fractions 20-45 were diluted 1:1 with distilled water and re-run onto the column of CM Sephadex C 25, washed with 0.1 mol/l phosphate buffer, pH 7, and the active fractions eluted with 0.1 mol/l NaOH. These fractions were adjusted to pH 1.7, evaporated to 5 ml and chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl. The results are shown in Fig. 6-36. Samples of 1 ml were taken from alternate fractions from 10 to 18 and freeze-dried. These were re-dissolved in SDS sample buffer and separated by SDS electrophoresis. The results are shown in Plate 6-15. A rather complex pattern was seen for fractions 10 and 12, but fractions 14, 16 and 18 appeared to be composed of single major bands.

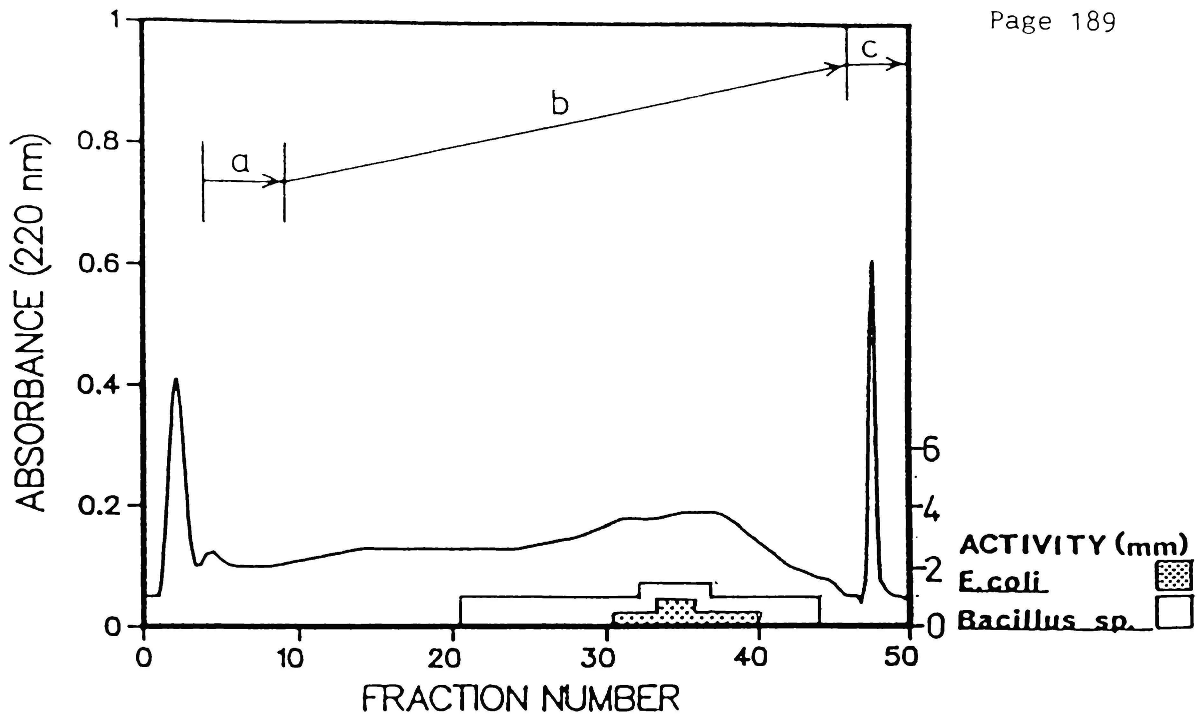


Fig. 6-35: Ion Exchange Chromatography of Fractions 11-14 from Fig. 6-25
Gel: CM Sephadex C 25
Buffer: 0.1 mol/l phosphate, pH 7
Sample: Fractions 11-14 (Fig. 6-25) adjusted to pH 7
Elution Buffers: a- 0.1 mol/l phosphate, pH 7; b- salt gradient from 0 to 1 mol/l NaCl in 0.1 mol/l phosphate buffer, pH 7; c- 0.1 mol/l NaOH
Fraction Volume: 10 ml

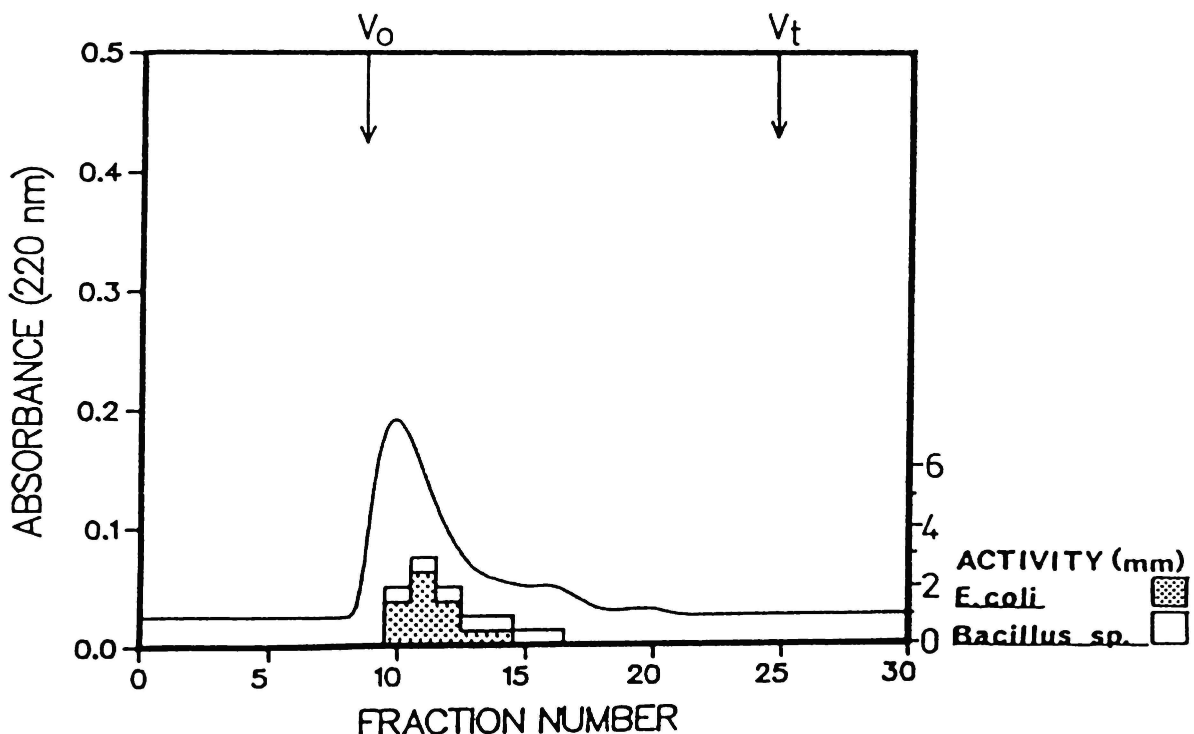


Fig. 6-36: Gel Filtration Chromatography of the Active Fractions from Fig. 6-35
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Active fractions (Fig. 6-35), desalted, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

Plate 6-15: SDS Electrophoresis

1 - not relevant

2 - not relevant

3 - not relevant

4 - not relevant

5 - fraction 18 from Fig. 6-36

6 - fraction 16 from Fig. 6-36

7 - fraction 14 from Fig. 6-36

8 - fraction 12 from Fig. 6-36

9 - fraction 10 from Fig. 6-36

10- α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin standards

Plate 6-16: Cationic Electrophoresis

1 - not relevant

2 - fraction 20 from Fig. 6-30

3 - fraction 18 from Fig. 6-30

4 - fraction 15 from Fig. 6-29

5 - fraction 15 from Fig. 6-34

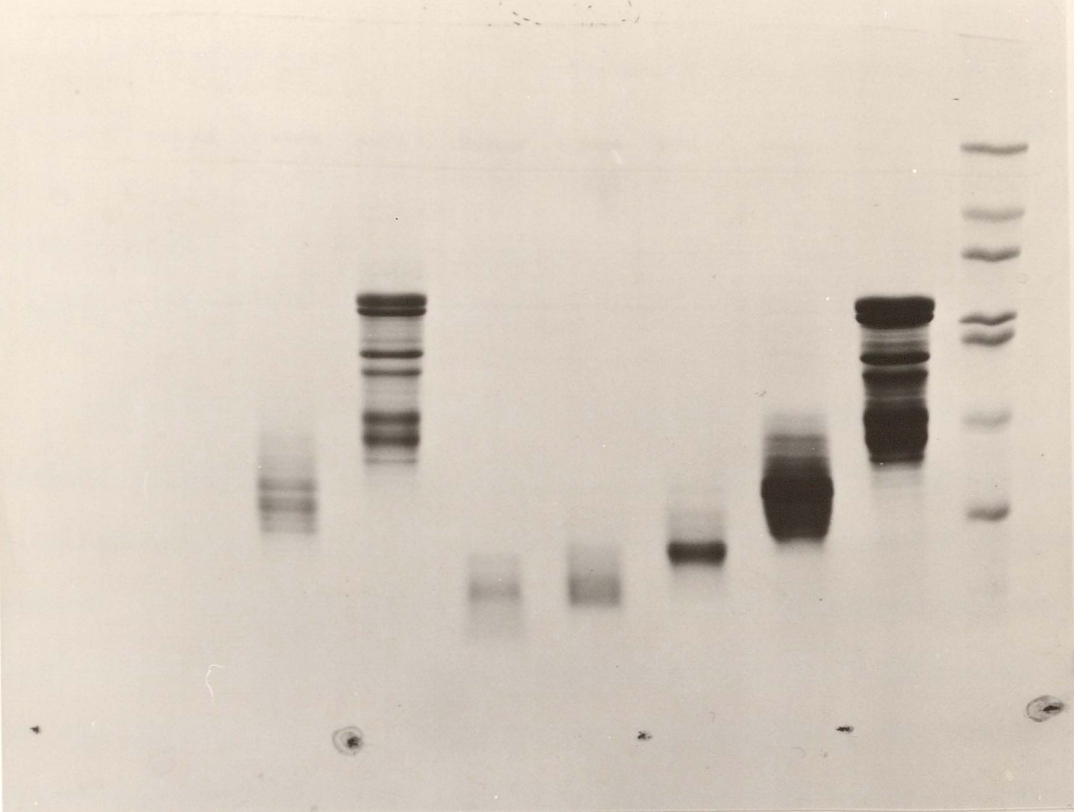
6 - fraction 12 from Fig. 6-33

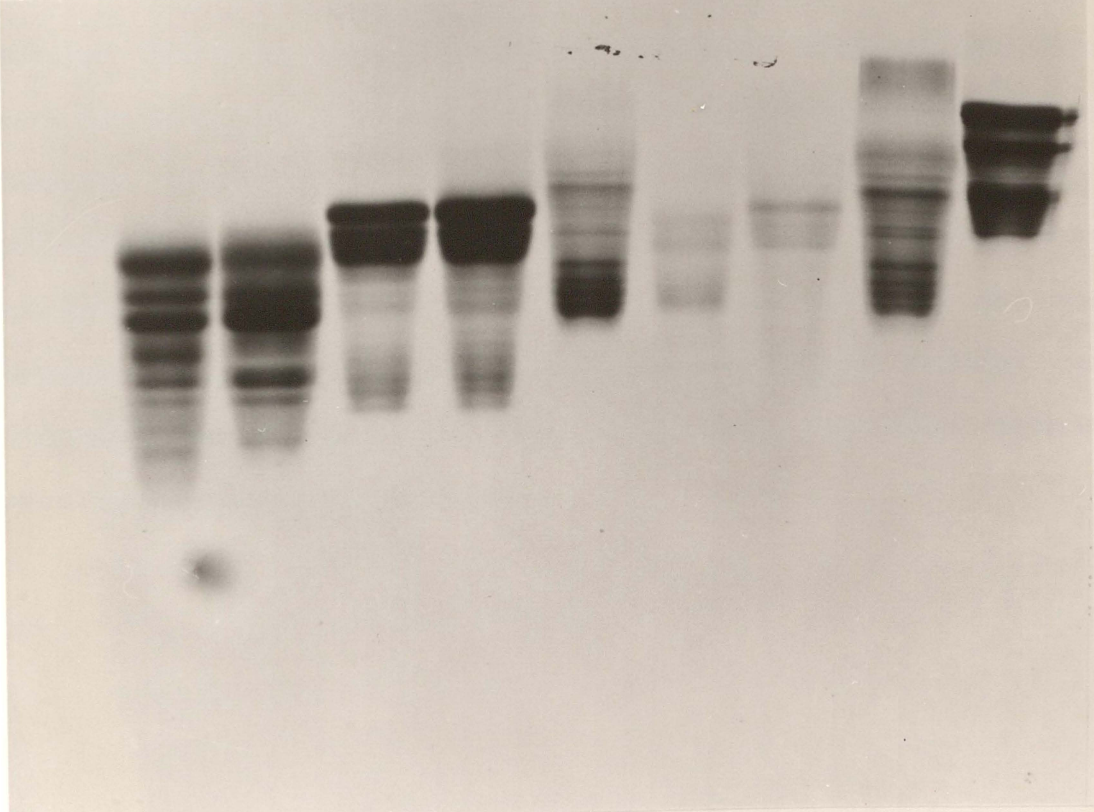
7 - fraction 16 from Fig. 6-36

8 - fraction 14 from Fig. 6-36

9 - fraction 12 from Fig. 6-36

10- fraction 10 from Fig. 6-36


1 2 3 4 5 6 7 8 9 10
−
+


1 2 3 4 5 6 7 8 9 10
+
−

It appeared from the results of SDS electrophoresis that the EDTA had helped considerably in dissociating the compounds of lower molecular weight as several single bands were obtained. Some of these bands were broad and overloaded, however the small size and cationic nature of these compounds may cause broadening of the bands by affecting the binding of SDS to the proteins.

Therefore it was necessary to examine these proteins using a different electrophoretic method. The previous samples run on SDS electrophoresis were prepared and run on cationic electrophoresis by the method of Reisfeld _et al_. (1962). A very different picture emerged (see Plates 6-16) which showed that there were still many bands present in the purest sample. Comparison of the results of SDS electrophoresis before and after dialysis indicated that the EDTA had some effect on the thymus preparation (compare Plates 6-1 and 6-2 with Plates 6-13 and 6-14). However complete dissociation of the antibacterial components from the thymus preparation was not achieved. It would appear that on SDS electrophoresis the single bands represent several peptides of similar molecular weight migrating at similar rates and overlapping to give the broadened bands. On cationic electrophoresis these compounds separate according to differences in their charge and size and hence multiple bands are obtained. However it is also possible that the multiple bands seen on cationic electrophoresis are the result of re-aggregation. Similar purification procedures were carried out for fractions 16-19 and 20-25 (Fig. 6-26) with the same results: no adequate separation was achieved.

6.7 Further Purification Methods Attempted.

Due to the lack of separation achieved by gel filtration and ion exchange chromatography, hydroxyapatite adsorption chromatography was used as a separation method. The sheep thymus preparation (50 mg) was dissolved in 20 ml of 10 mmol/l phosphate buffer pH 7 and run onto a column of hydroxyapatite equilibrated with the same buffer. After establishing a stable baseline the column was eluted with a gradient from 10 mmol/l to 400 mmol/l phosphate buffer, pH 7, followed by a final wash with 2.0 mol/l NaCl. The results are shown in Fig. 6-37. Antibacterial activity was found throughout the elution profile and it appeared little separation had occurred. Fractions 3-13, 14-24, 25-84 and 85-87 were separately pooled. Fractions 14-24 were found by thin-layer chromatography to be spermine and spermidine. They had not been removed from the thymus preparation before hand as had been done in previous occasions.

Fractions 85-87 were evaporated to 10 ml, adjusted to pH 1.7 with HCl and chromatographed through a column of Sephadex G 50 eluted with 0.02 mol/l HCl. The resulting elution profile (Fig. 6-38) was composed of 4 major active peaks (fractions 10-11, 12-15, 16-18, 19-22 and 23-26).

Fractions 25-84 (Fig. 6-37) were pooled and diluted 1:1 with distilled water and eluted through a column of CM Sephadex C 25 equilibrated with 0.1 mol/l phosphate buffer, pH 7. The active fractions were then eluted with 0.1 mol/l NaOH, adjusted to pH 1.7 with HCl, evaporated to 10 ml and chromatographed through a column of Sephadex G 50 eluted with 0.02 mol/l HCl. The elution profile was essentially the same as in Fig. 6-38 (with slight variations in peak

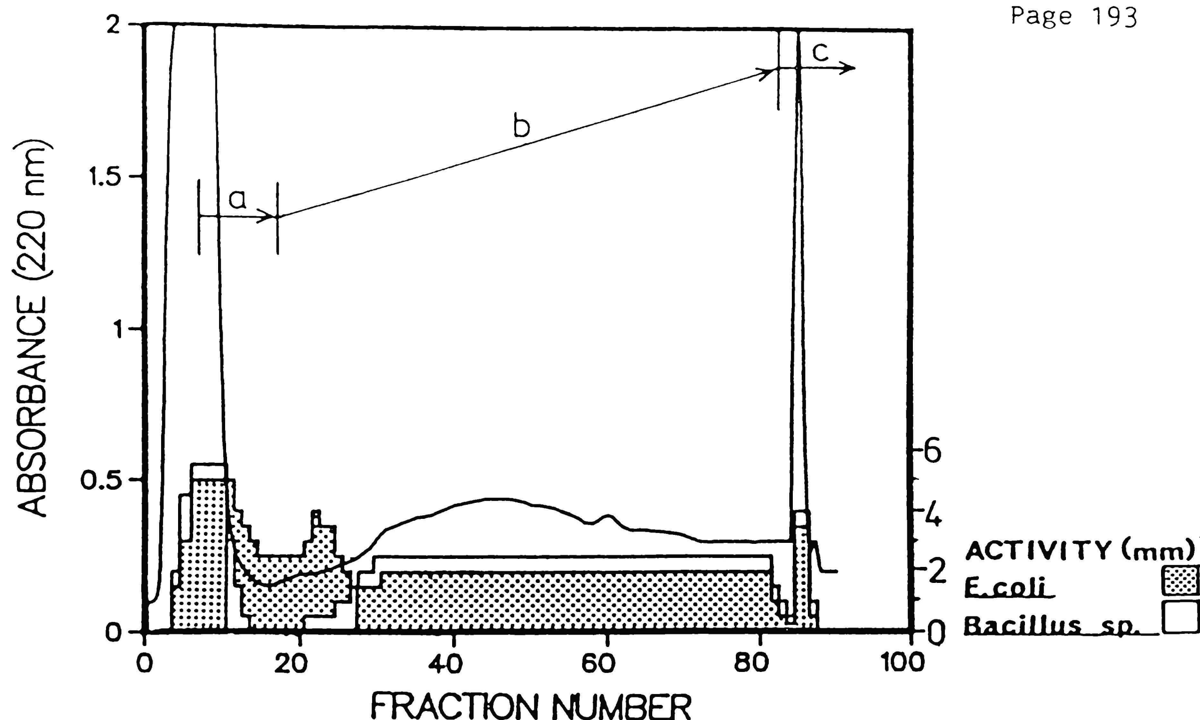


Fig. 6-37: Adsorption Chromatography of the Sheep Thymus Preparation
Gel: Hydroxyapatite Bio-Gel HTP
Buffer: 10 mmol/l phosphate, pH 7
Sample: Sheep thymus preparation dissolved in 10 mmol/l phosphate buffer, pH 7
Elution Buffers: a- 10 mmol/l phosphate buffer, pH 7; b- gradient from 10 to 400 mmol/l phosphate buffer, pH 7; c- 2 mol/l NaCl
Fraction Volume: 10 ml

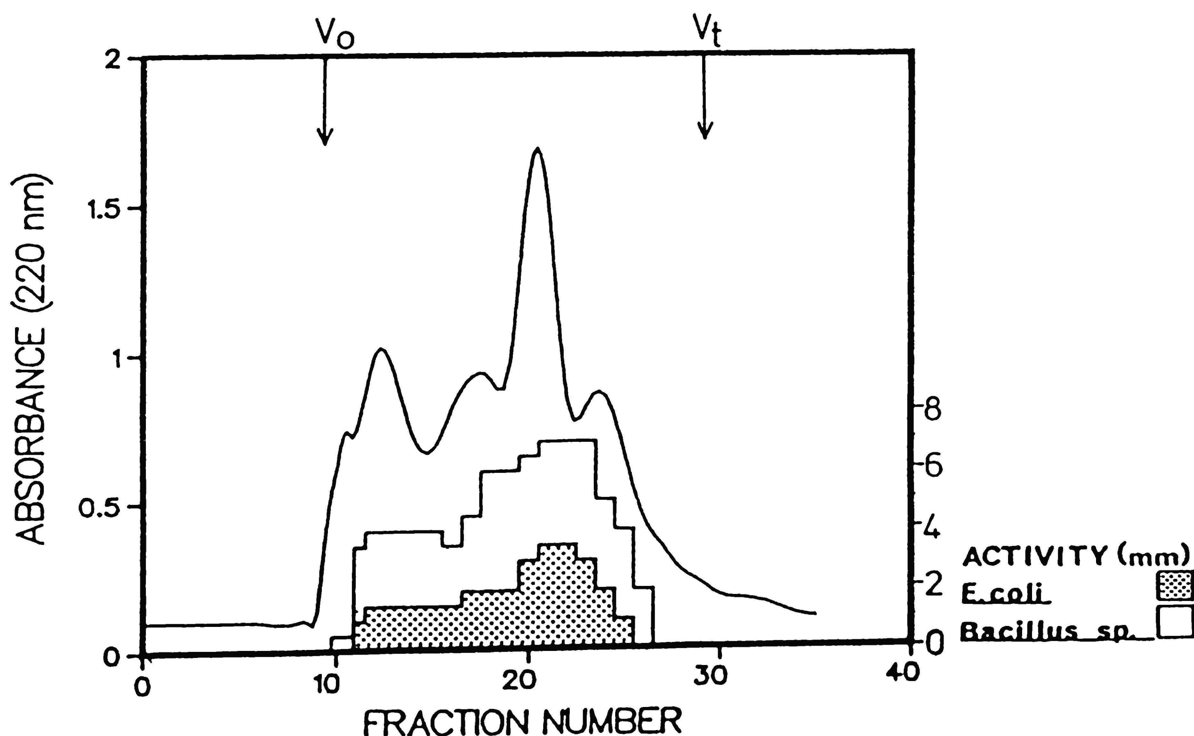


Fig. 6-38: Gel Filtration Chromatography of Fractions 85-87 from Fig. 6-37
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 85-87 (Fig. 6-37) evaporated to 10 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

height) and the fractions were pooled in the same manner. Thus the peak eluted with the 2.0 mol/l NaCl was probably the tail of what had been eluted with the salt gradient.

Fractions 3-13 (Fig. 36) were also evaporated to 10 ml, adjusted to pH 1.7 and chromatographed on the column of Sephadex G 50. Again a similar elution profile to that shown in Fig. 6-38 resulted and the fractions were pooled accordingly. The hydroxyapatite did not give any separation of the activity chromatographed on it, but the material chromatographed differently on Sephadex G 50 after passage through the hydroxyapatite (compare Fig. 6-38 with Figs. 6-8 and 6-25). The corresponding pooled fractions from each of the runs through the column of Sephadex G 50 were combined for further purification.

In a further attempt to isolate the active components they were chromatographed in the presence of 6 mol/l urea to obtain further disaggregation.

The pooled fractions 10-11 from the three runs were evaporated to dryness, re-dissolved in 5 ml of 6 mol/l urea and chromatographed on a Sephadex G 50 column eluted with 6 mol/l urea. The results are shown in Fig 6-39. Fractions 10-11, 12-14 and 30-33 were separately pooled and run onto a column of CM Sephadex C 25, washed with 0.1 mol/l phosphate buffer, pH 7, and the protein removed with 0.1 mol/l NaOH. The fractions were neutralised and assayed for antibacterial activity. Both pooled fractions 10-11 and 12-14 were active. These active fractions were separately evaporated to 5 ml and chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl. The results are shown in Figs. 6-40 and 6-41. Samples of 1 ml were taken from fraction 12 (Fig. 6-40) and fractions 11 and 14 (Fig. 6-41), freeze-dried,

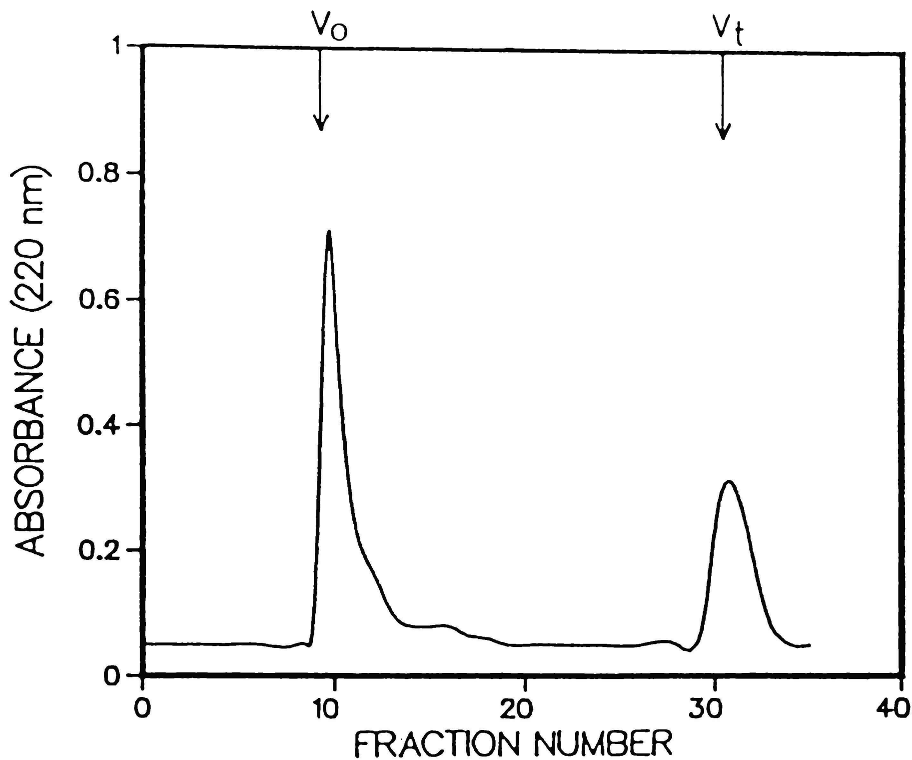


Fig. 6-39: Gel Filtration Chromatography of Fractions 10-11 from Fig. 6-38
Gel: Sephadex G 50 F
Eluent: 6 mol/l urea
Sample: Fractions 10-11 (Fig. 6-38) evaporated to dryness and re-dissolved in 5 ml of 6 mol/l urea
Fraction Volume: 10 ml

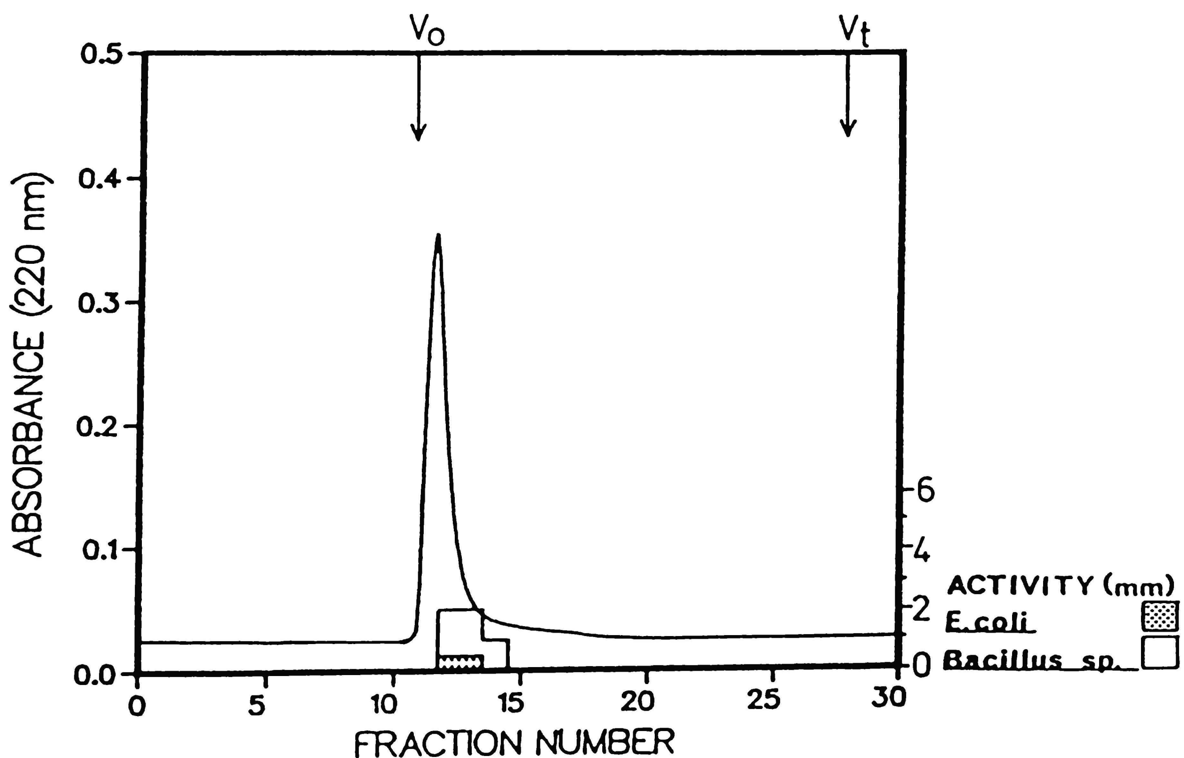


Fig. 6-40: Gel Filtration Chromatography of Fractions 10-11 from Fig. 6-39
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 10-11 (Fig. 6-39), urea removed, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

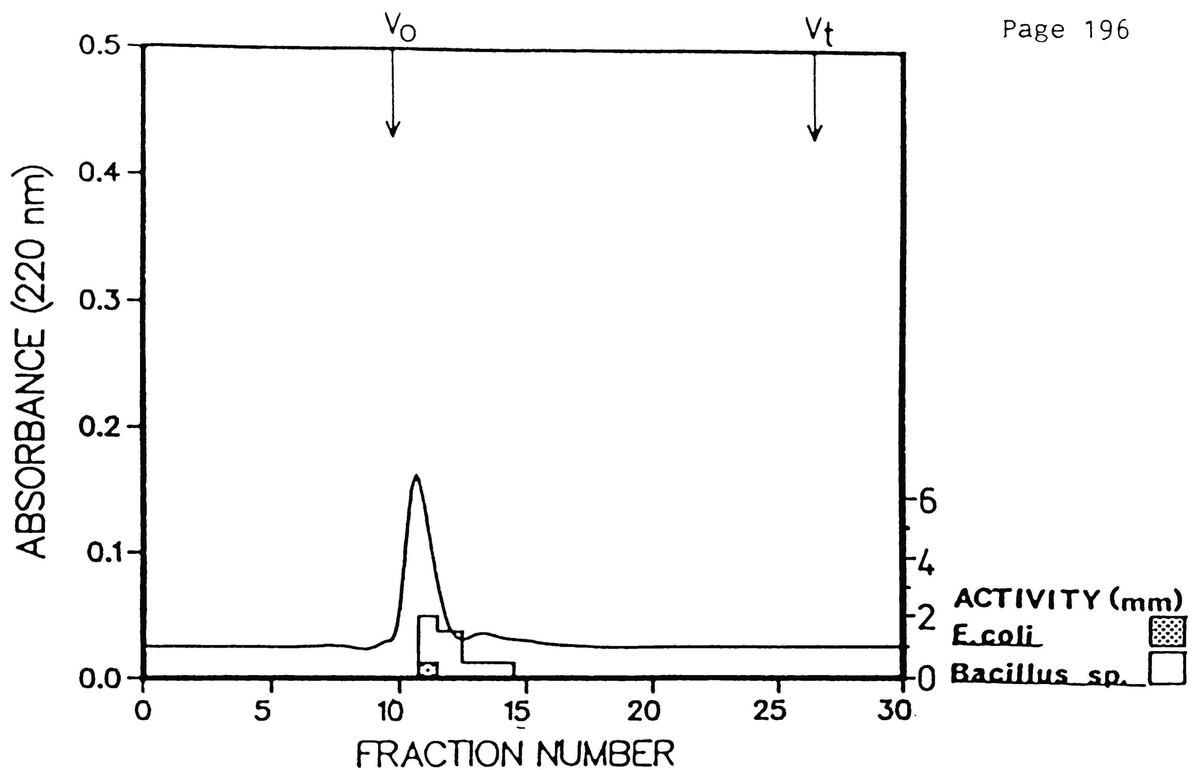


Fig. 6-41: Gel Filtration Chromatography of Fractions 12-14 from Fig. 6-39
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 12-14 (Fig. 6-39), urea removed, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

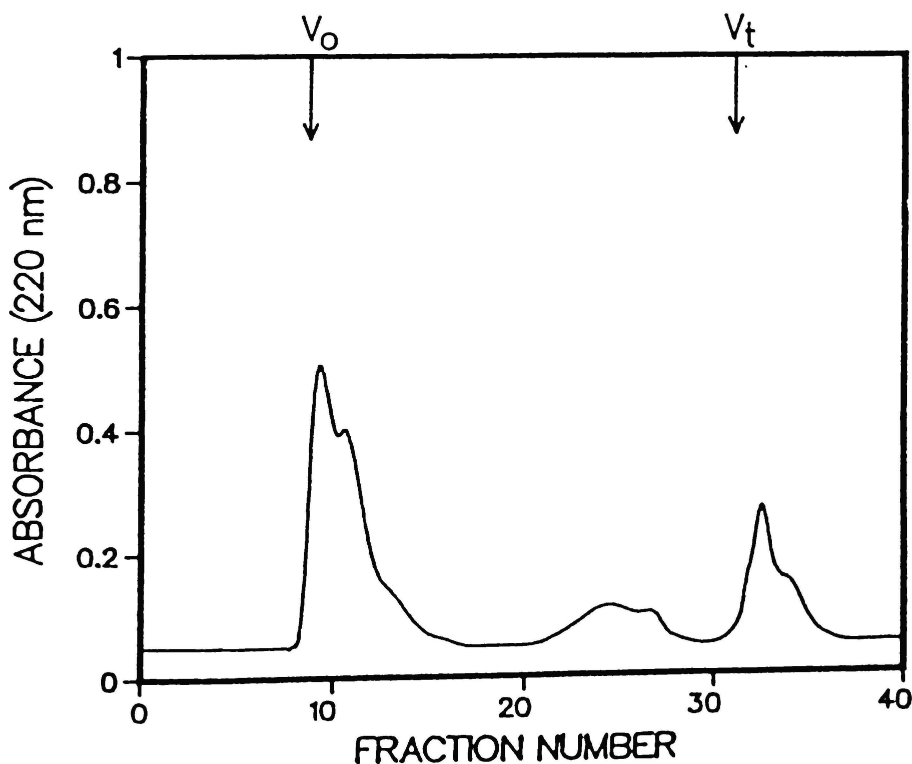


Fig. 6-42: Gel Filtration Chromatography of Fractions 12-15 from Fig. 6-38
Gel: Sephadex G 50 F
Eluent: 6 mol/l urea
Sample: Fractions 12-15 (Fig. 6-38) evaporated to dryness and re-dissolved in 5 ml of 6 mol/l urea
Fraction Volume: 10 ml

re-dissolved in sample buffer and examined on cationic electrophoresis. The results are shown in Plate 6-17. Fraction 12 (Fig. 6-40) contained a major band which had a migration similar to that of the egg white lysozyme standard. Fraction 11 (Fig. 6-41) also had this band present along with 2 other major bands. Fraction 12 was also found to lyse pre-grown Micrococcus lysodeikticus cells, further indicating the presence of bovine lysozyme. A sample (1 ml) of fraction 12 (Fig. 6-40) was freeze-dried, re-dissolved in SDS sample buffer and separated by SDS electrophoresis (see Plate 6-18). The major band present had a mobility similar to that of the lysozyme-like protein isolated from bovine seminal plasma. Its molecular weight was estimated to be 15 000.

The pooled fractions 12-15 from the three runs in Fig. 6-38 were evaporated to dryness and re-dissolved in 6 mol/l urea and chromatographed on a column of Sephadex G 50 eluted with 6 mol/l urea. The results are shown in Fig. 6-42. Fractions 9-10, 11-15, 22-28 and 32-36 were separately pooled and each pool run through a column of CM Sephadex C 25. The column was washed with 0.1 mol/l phosphate buffer, pH 7, and the bound protein removed with 0.1 mol/l NaOH. After neutralising they were assayed for antibacterial activity which was detected in fractions 9-10 and 11-15.

Fractions 9-10 and 11-15 were separately evaporated to 5 ml and chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl. The results are shown in Figs. 6-43 and 6-44. Samples of 1 ml were taken from fraction 12 (Fig. 6-43) and fractions 13 and 15 (Fig. 6-44), freeze-dried, re-dissolved in SDS sample buffer and separated by SDS electrophoresis. The results are shown in Plate 6-19. Fraction 12 (Fig. 6-43) had several bands present, with a major band with the same

Plate 6-17: Cationic Electrophoresis

1 - fraction 12 from Fig. 6-40

2 - fraction 11 from Fig. 6-41

3 - fraction 14 from Fig. 6-41

4 - fraction 21 from Fig. 6-49

5 - fraction 24 from Fig. 6-49

6 - fraction 25 from Fig. 6-52

7 - fraction 14 from Fig. 6-46

8 - fraction 16 from Fig. 6-46

9 - fraction 18 from Fig. 6-46

10- lysozyme standard

Plate 6-18: SDS Electrophoresis

1 - α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin standards

2 - lysozyme-like protein from seminal plasma

3 - not relevant

4 - not relevant

5 - fraction 12 from Fig. 6-40

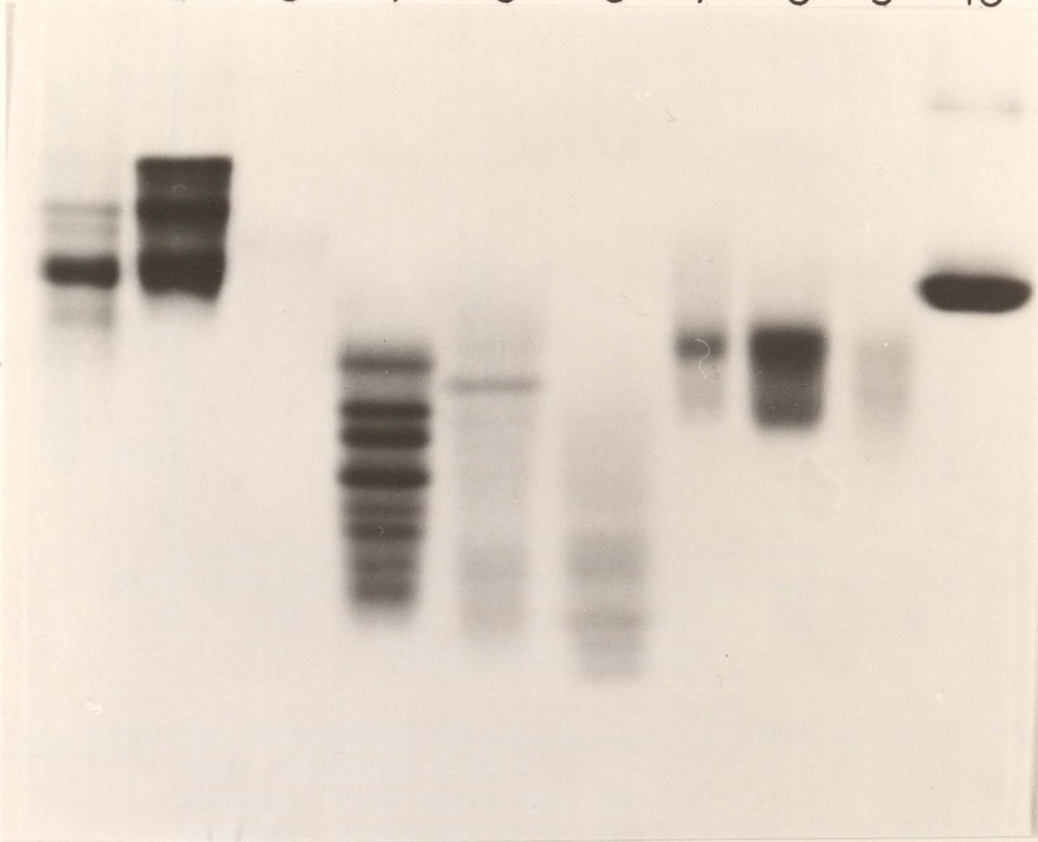
6 - not relevant

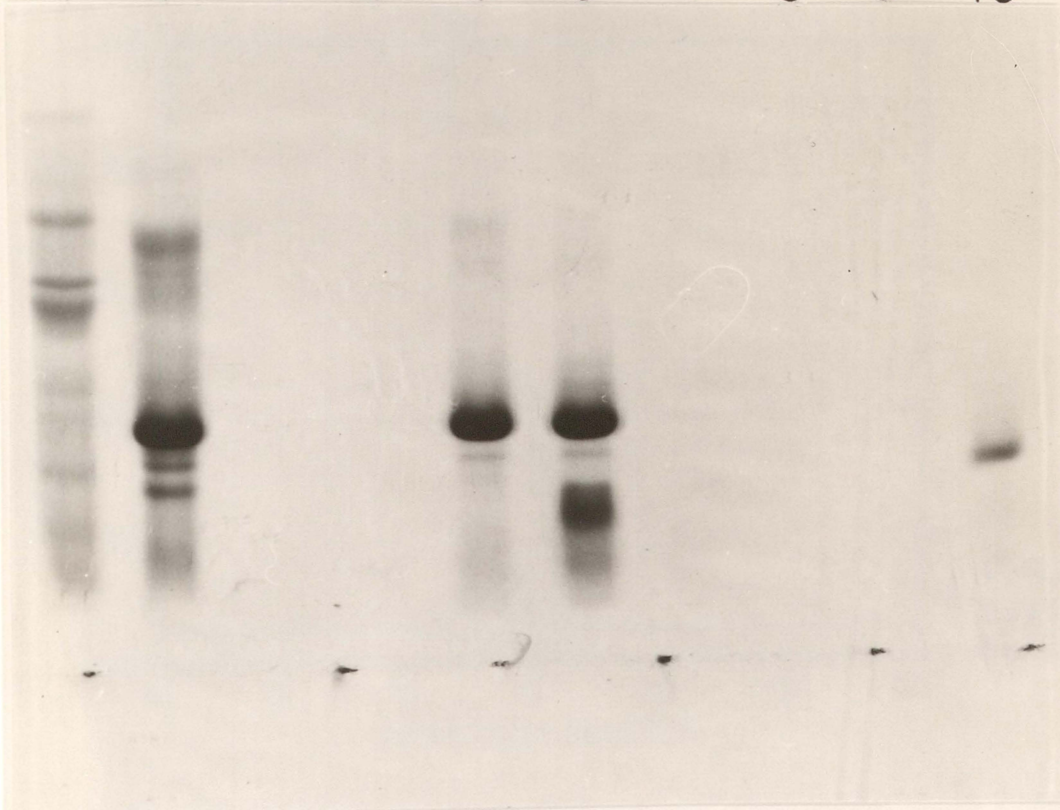
7 - not relevant

8 - not relevant

9 - not relevant

10- α-lactalbumin standard


1 2 3 4 5 6 7 8 9 10
+
−


1 2 3 4 5 6 7 8 9 10
−
+

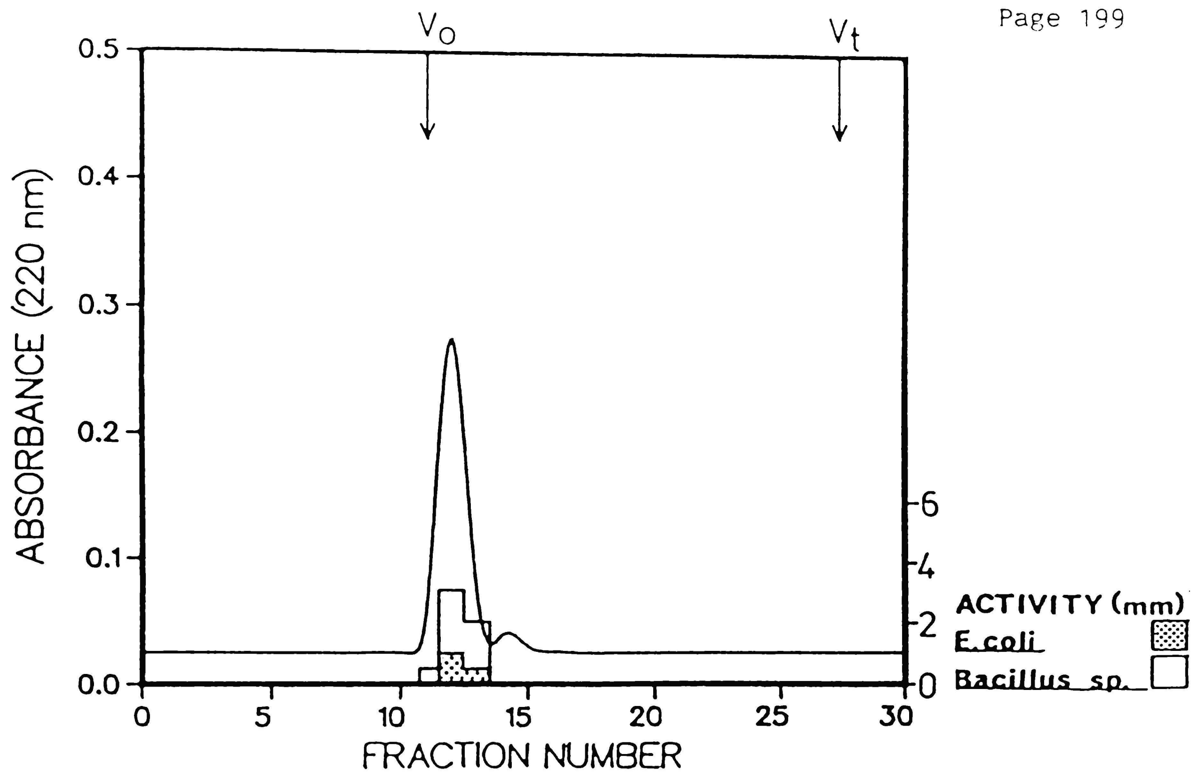


Fig. 6-43: Gel Filtration Chromatography of Fractions 9-10 from Fig. 6-42
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 9-10 (Fig. 6-42), urea removed, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

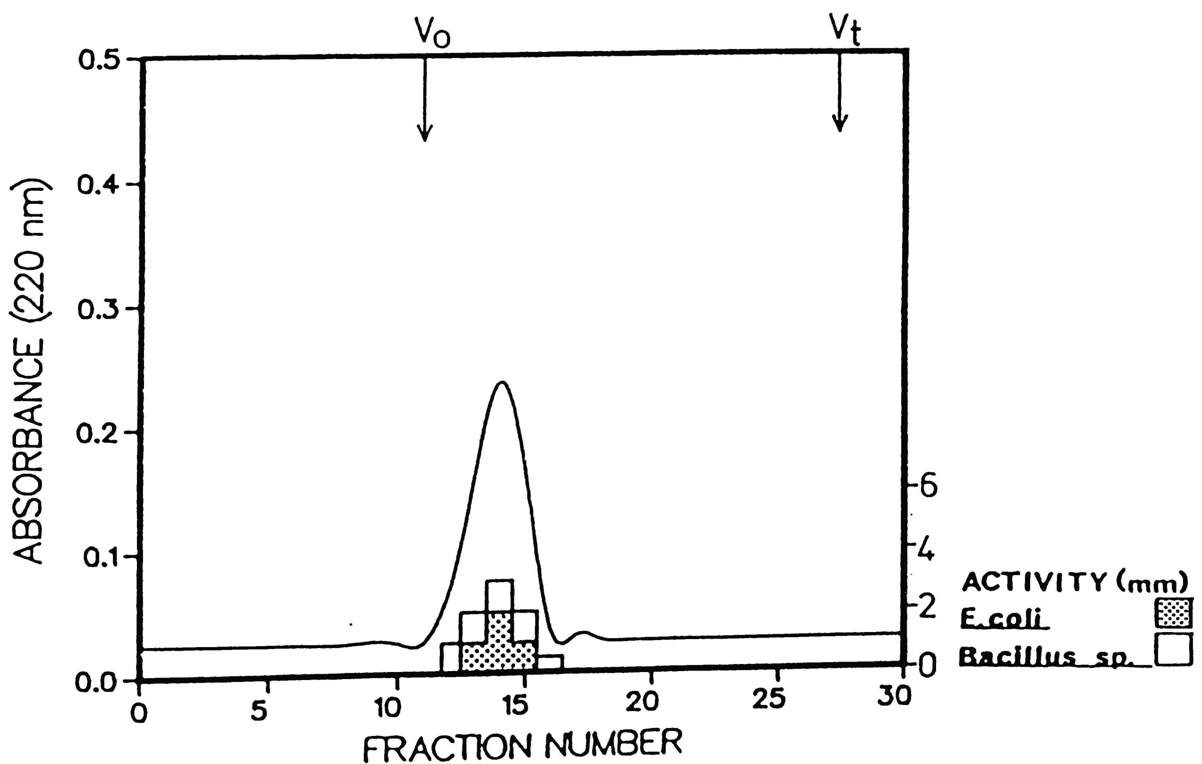


Fig. 6-44: Gel Filtration Chromatography of Fractions 11-15 from Fig.6-42
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 11-15 (Fig. 6-42), urea removed, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

Plate 6-19: SDS Electrophoresis

1 - α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin standards

2 - not relevant

3 - not relevant

4 - not relevant

5 - not relevant

6 - fraction 15 from Fig. 6-44

7 - fraction 13 from Fig. 6-44

8 - fraction 12 from Fig. 6-33

9 - not relevant

10- lysozyme, β-lactoglobulin and trypsinogen standards

Plate 6-20: SDS Electrophoresis

1 - fraction 23 from Fig. 6-52

2 - fraction 25 from Fig. 6-52

3 - fraction 27 from Fig. 6-52

4 - not relevant

5 - fraction 14 from Fig. 6-46

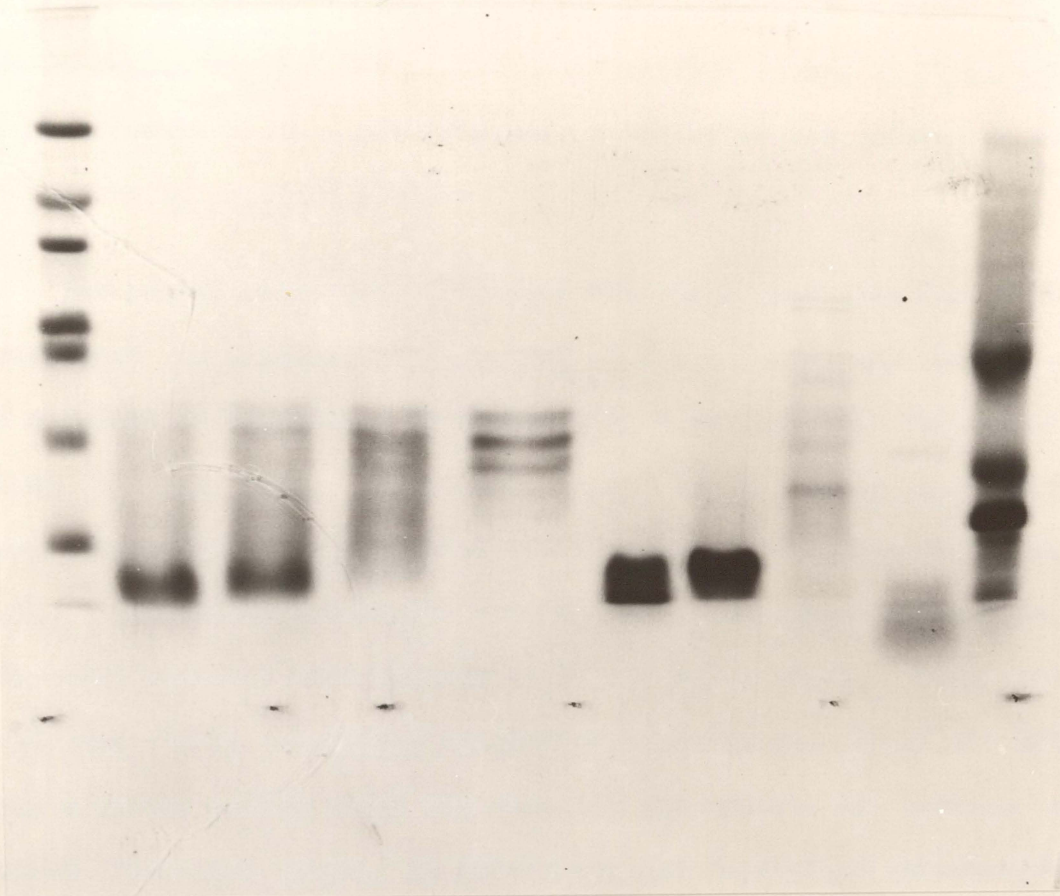
6 - fraction 16 from Fig. 6-46

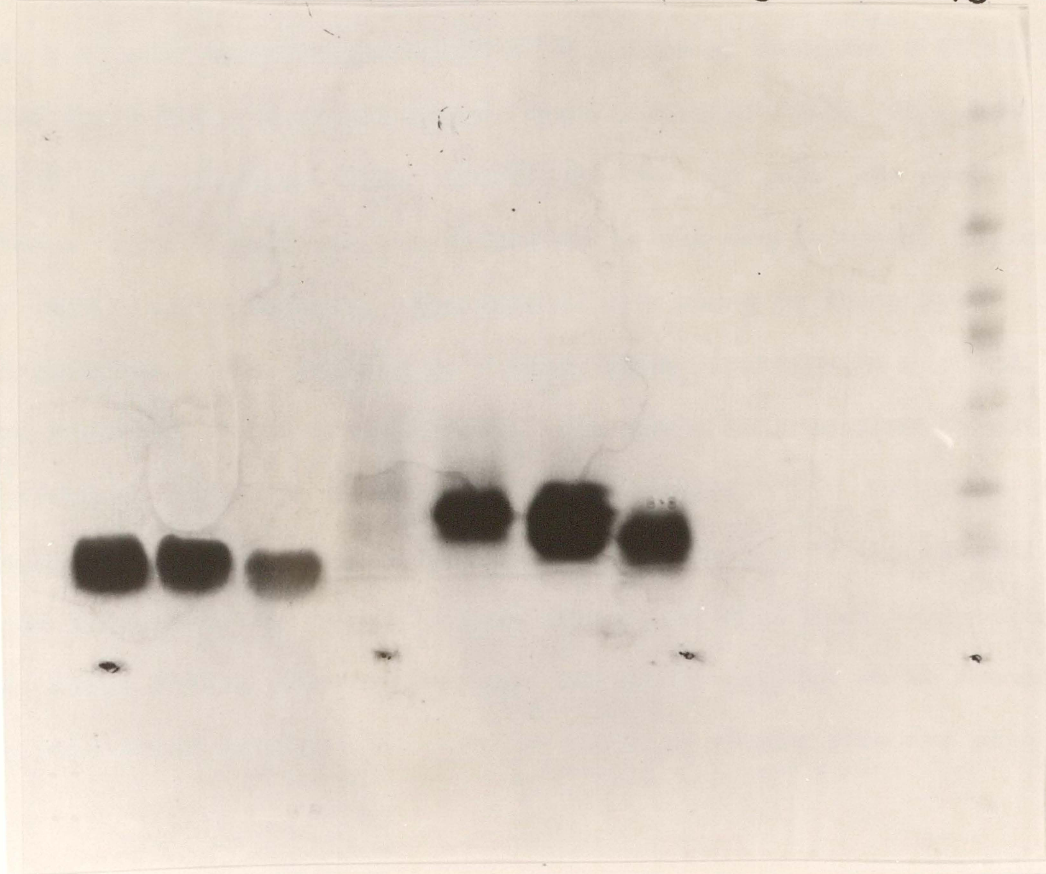
7 - fraction 18 from Fig. 6-46

8 - not relevant

9 - not relevant

10- α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin standards


1 2 3 4 5 6 7 8 9 10
−
+


1 2 3 4 5 6 7 8 9 10
−
+

migration as that calculated for the lysozyme-like protein found in pooled fractions 10-11 (Fig. 6-38). Fractions 13 and 15 (Fig. 6-44) ran as broad bands which might have been a result of the irregular binding of the SDS to cationic proteins.

The pooled fractions 16-18 from the three runs (see Fig. 6-38) were evaporated to dryness, re-dissolved in 6 mol/l urea and chromatographed on a column of Sephadex G 50 eluted with 6 mol/l urea. The results are shown in Fig. 6-45. Fractions 9-12, 13-16, 25-26 and 28-30 were separately pooled and each pool run through a column of CM Sephadex C 25. The column was washed with 0.1 mol/l phosphate buffer, pH 7, and the bound proteins were removed with 0.1 mol/l NaOH. Only fractions 9-12 and 13-16 contained antibacterial activity. Fractions 9-12 were only slightly active which was possibly the result of overlap of the active fractions 13-16. In subsequent gel filtration chromatography of fractions 9-12, no activity was detected in the peak obtained.

Fractions 13-16 (Fig. 6-45), with the urea removed, were evaporated to 5 ml and re-chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl. A single major peak resulted (see Fig. 6-46), from which 1 ml samples from fractions 14, 16 and 18 were taken and freeze-dried. They were re-dissolved in SDS sample buffer and separated by SDS electrophoresis. The results are shown in Plate 6-20. Fraction 16 appeared to be composed of 1 major band, fraction 16 had two bands, and fraction 18 had 1 major band. The bands in fractions 14 and 18 were different, and both were found in fraction 16. Activity must be associated with both bands as both fractions 14 and 18 were active. When fractions 14, 16 and 18 were run on cationic electrophoresis (shown in Plate 6-17), there did not obviously appear to be two different antibacterial agents. The band in fraction 14 was also the major band

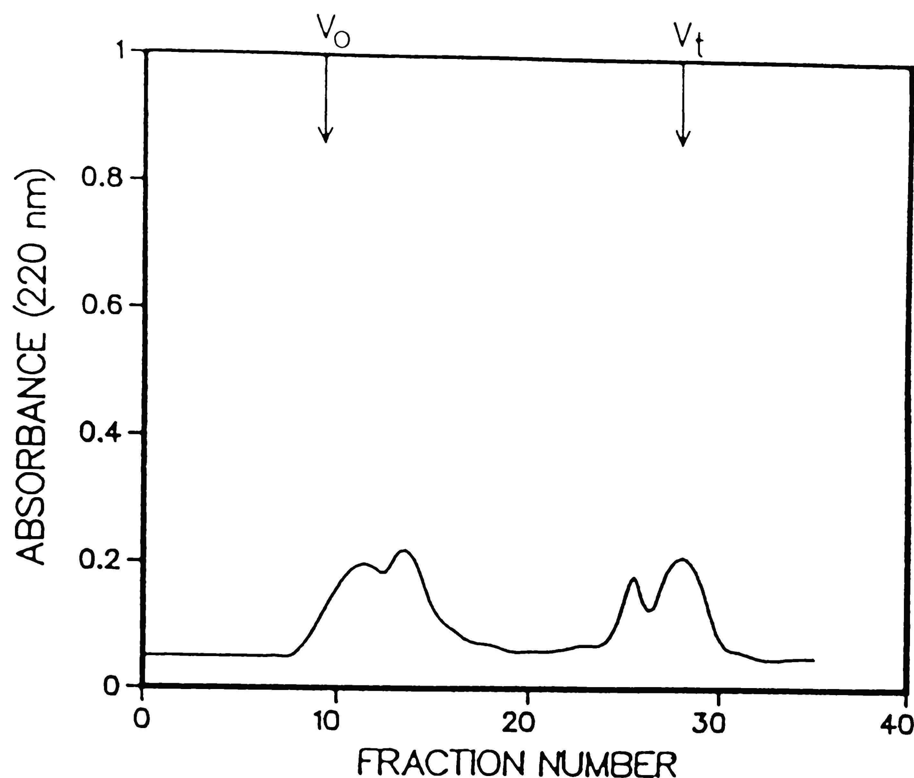


Fig. 6-45: Gel Filtration Chromatography of Fractions 16-18 from Fig. 6-38
Gel: Sephadex G 50 F
Eluent: 6 mol/l urea
Sample: Fractions 16-18 (Fig. 6-38), evaporated to dryness and re-dissolved in 5 ml of 6 mol/l urea
Fraction Volume: 10 ml

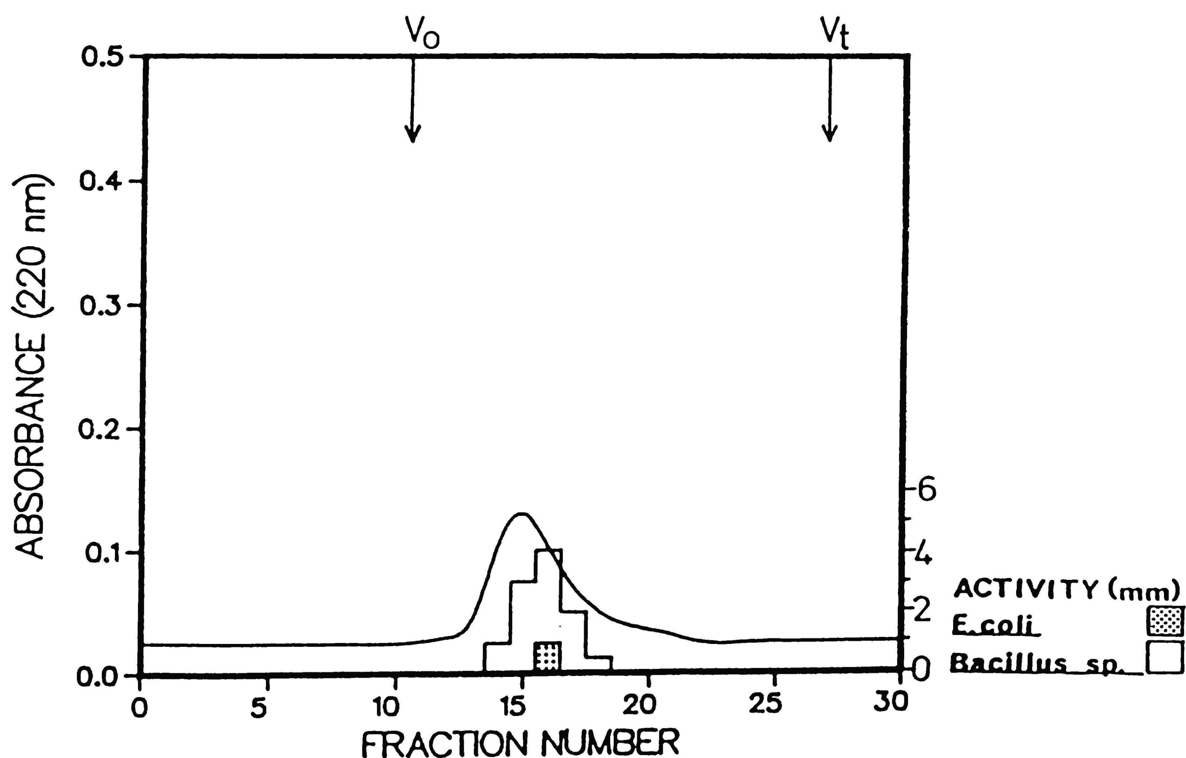


Fig. 6-46: Gel Filtration Chromatography of Fractions 13-16 from Fig. 6-45
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 13-16 (Fig. 6-45), urea removed, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

in fraction 16 and it was present in fraction 18 also, although it was indistinct.

The pooled fractions 19-22 (from the three runs as in Fig. 6-38) were evaporated to dryness and re-dissolved in 6 mol/l urea and chromatographed on a column of Sephadex G 50 eluted with 6 mol/l urea. The results are shown in Fig. 6-47. Four major peaks were eluted (fractions 13-17, 18-24, 30-31 and 33-36). These pools were separately run onto a column of CM Sephadex C 25, washed with 0.1 mol/l phosphate buffer, pH 7, and the protein eluted from the column with 0.1 mol/l NaOH. The fractions were assayed for antibacterial activity and fractions 13-17 and 18-24 were found to be active.

The active fractions 13-17 were evaporated to 5 ml, adjusted to pH 1.7 and chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl. A broad peak resulted (see Fig. 6-48) with antibacterial activity spread throughout. Samples of 0.5 ml were taken from fractions 18, 20 and 24, freeze-dried, re-dissolved in SDS sample buffer and separated by SDS electrophoresis. The results are shown in Plate 6-21. Fraction 18 was composed of 1 major band while fraction 20 had 3 bands present. Fraction 23 appeared to be the same as fraction 20 but had a lower protein concentration.

The active fractions 18-24 (Fig. 6-47) were evaporated to 5 ml, adjusted to pH 1.7 and chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl. Again a broad symmetrical peak resulted (see Fig. 6-49) with activity spread throughout. Samples of 0.5 ml were taken from fractions 18, 21 and 24, freeze-dried, re-dissolved in SDS sample buffer and separated by SDS electrophoresis as shown in Plate 6-21. Fractions 18 and 21 ran as very broad bands while fraction

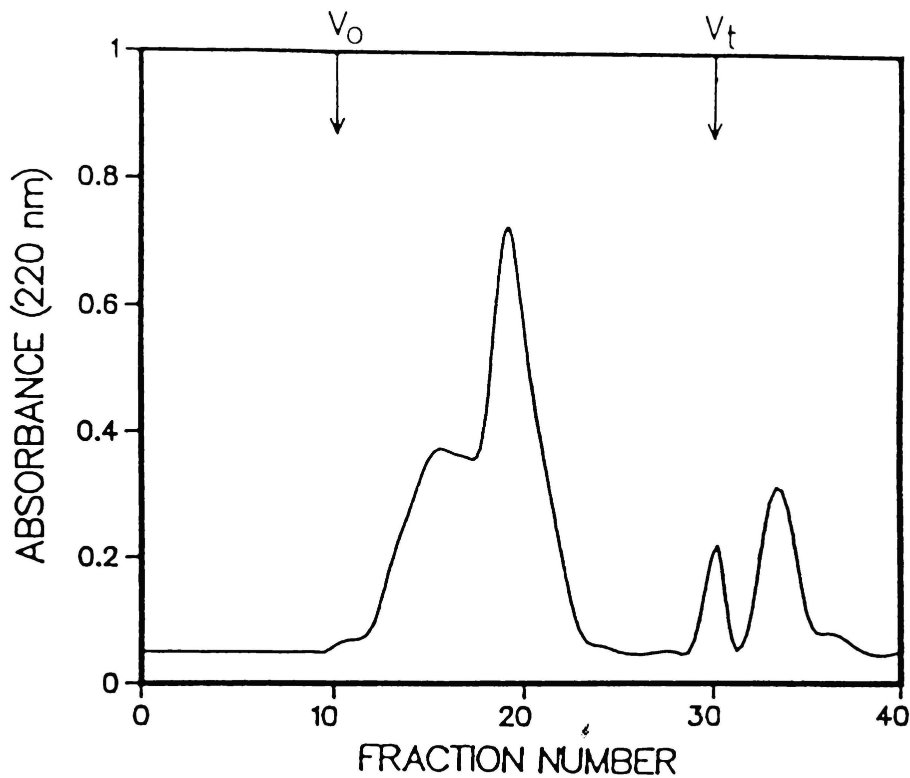


Fig. 6-47: Gel Filtration Chromatography of Fractions 19-22 from Fig. 6-38
Gel: Sephadex G 50 F
Eluent: 6 mol/l urea
Sample: Fractions 19-22 (Fig. 6-38), evaporated to dryness and re-dissolved in 5 ml of 6 mol/l urea
Fraction Volume: 10 ml

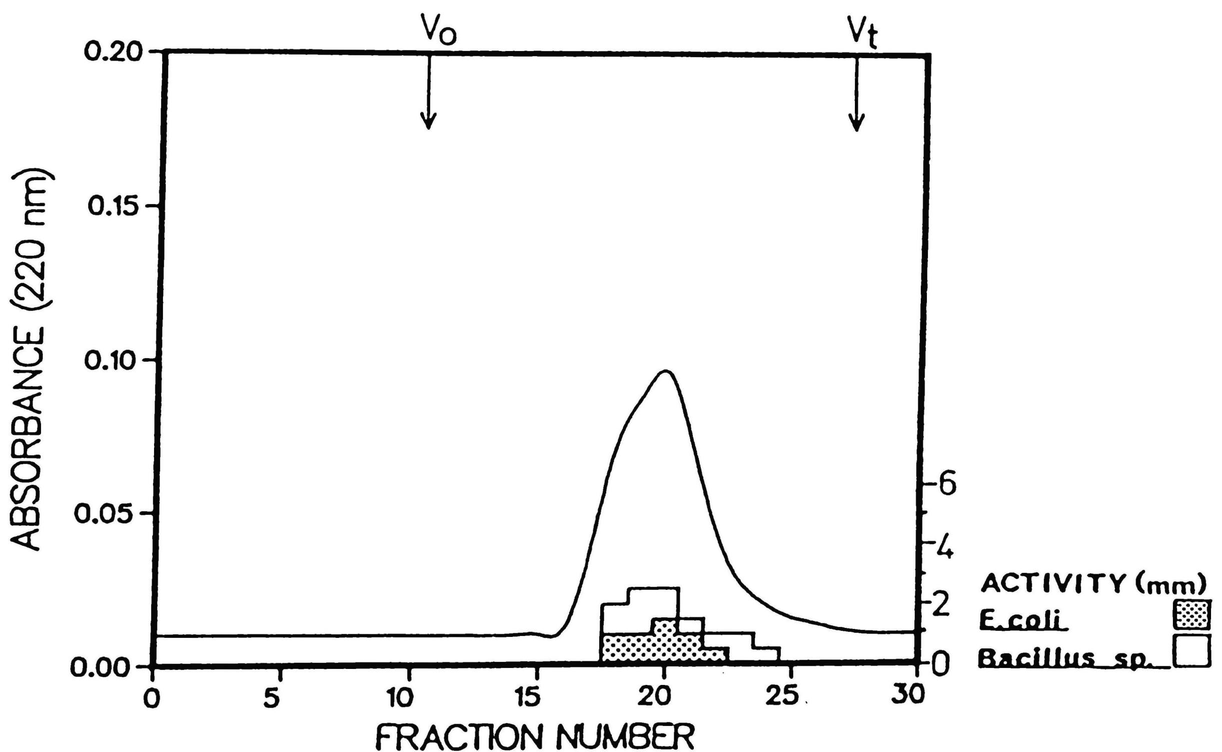


Fig. 6-48: Gel Filtration Chromatography of Fractions 13-17 from Fig. 6-47
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 13-17 (Fig. 6-47), urea removed, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

Plate 6-21: SDS Electrophoresis

1 - α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine serum albumin standards

2 - fraction 26 from Fig. 6-51

3 - fraction 23 from Fig. 6-48

4 - fraction 20 from Fig. 6-48

5 - fraction 18 from Fig. 6-48

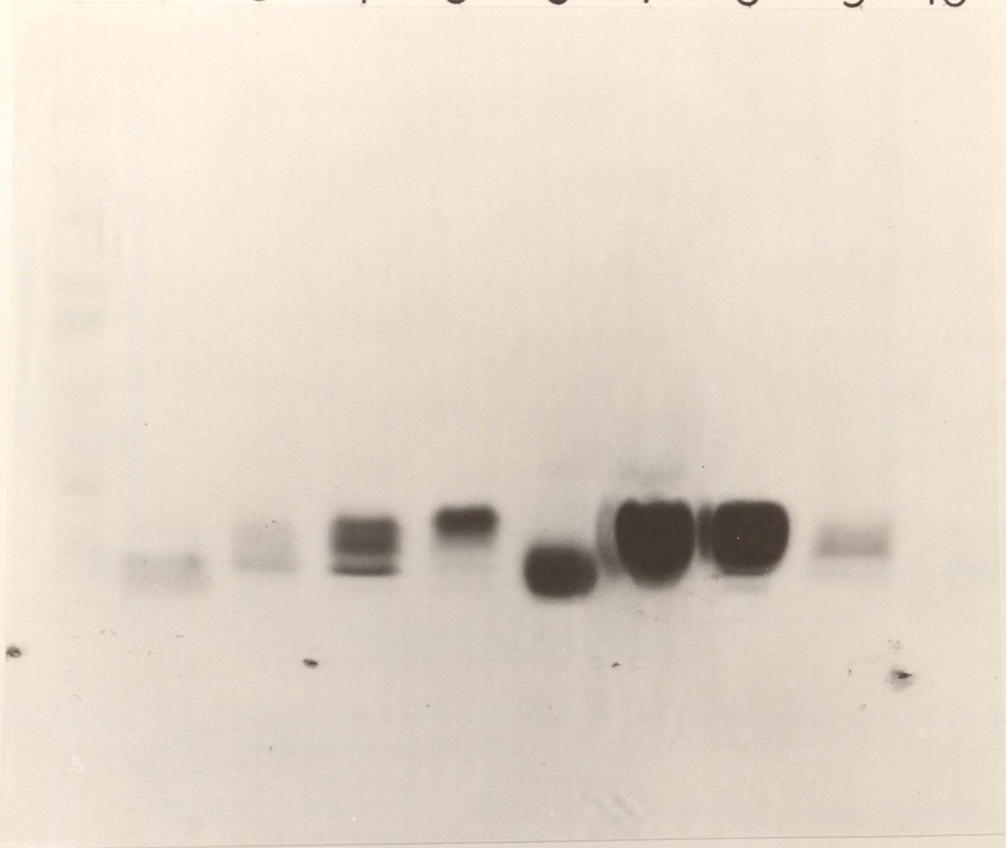
6 - fraction 24 from Fig. 6-49

7 - fraction 21 from Fig. 6-49

8 - fraction 18 from Fig. 6-49

9 - not relevant

10- not relevant


1 2 3 4 5 6 7 8 9 10
−
+

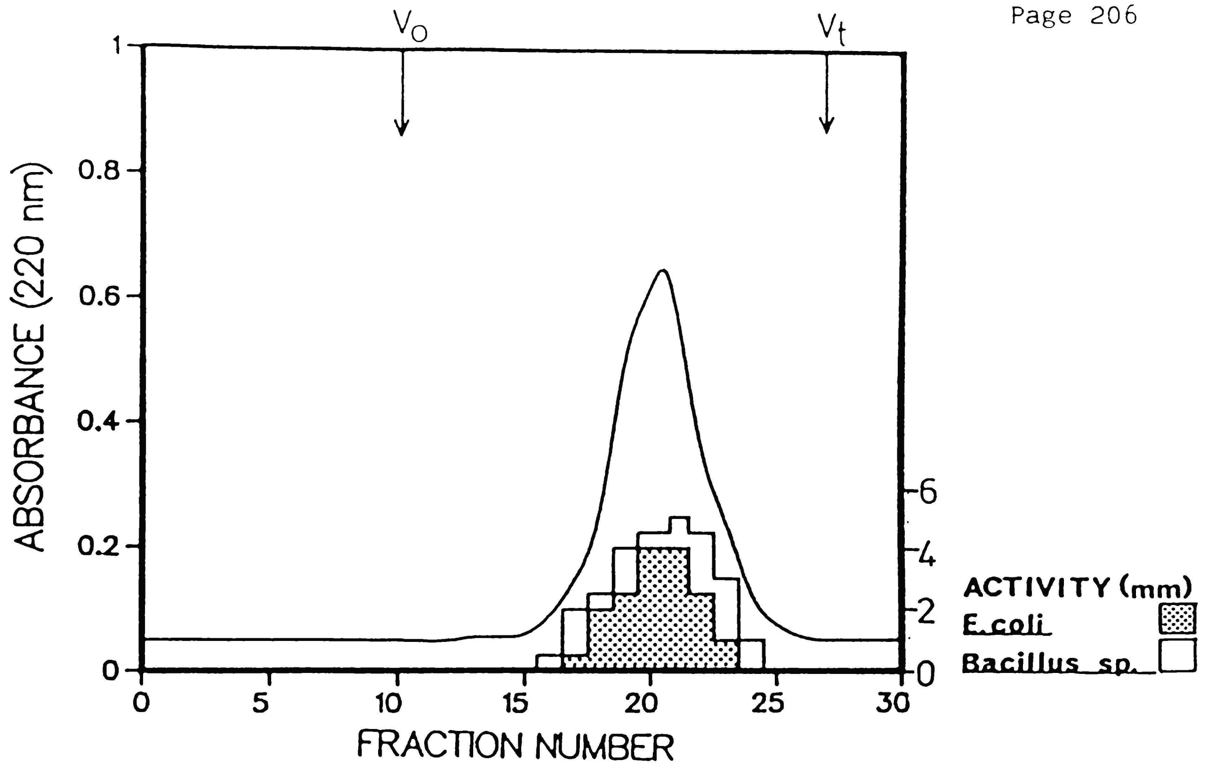


Fig. 6-49: Gel Filtration Chromatography of Fractions 18-24 from Fig. 6-47
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH1.7
Sample: Fractions 18-24 (Fig. 6-47), urea removed, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

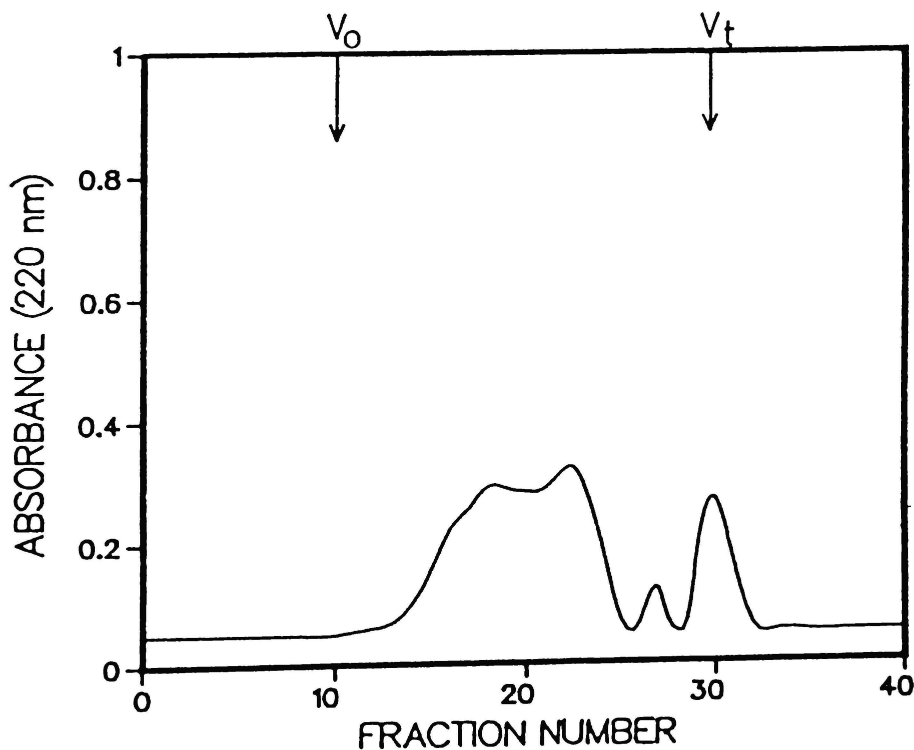


Fig. 6-50: Gel Filtration Chromatography of Fractions 23-26 from Fig. 6-38
Gel: Sephadex G 50 F
Eluent: 6 mol/l urea
Sample: Fractions 23-26 (Fig. 6-38), evaporated to dryness and re-dissolved in 5 ml of 6 mol/l urea
Fraction Volume: 10 ml

24 was not as broad and migrated further. These fractions had migration distances similar to those of fractions 18, 20 and 23 from the chromatography shown in Fig. 6-48. Presumably the active component were the same in both runs. Fraction 21 and 24 (Fig. 6-49) were prepared and separated by cationic electrophoresis (shown in Plate 6-17). Fraction 21 which had the majority of antibacterial activity contained 8 bands whereas it had only 1 major broad band on SDS electrophoresis.

The pooled fractions 23-26 (from the three runs as in Fig. 6-38) were evaporated to dryness, re-dissolved in 5 ml of 6 mol/l urea and chromatographed on a column on Sephadex G 50 eluted with 6 mol/l urea. The results are shown in Fig. 6-50. Four major peaks were eluted (fractions 15-20, 21-25, 27-28 and 30-32). The pooled fractions were separately run through a column of CM Sephadex C 25, washed with 0.1 mol/l phosphate buffer and the bound protein removed with 0.1 mol/l NaOH. These fractions were neutralised and assayed for antibacterial activity. Fractions 15-20 and 21-25 were found to be active although most of the activity was in fractions 21-25. Pooled fractions 15-20 and 21-25 were separately evaporated to 5 ml, adjusted to pH 1.7 and chromatographed on a column of Sephadex G 50 eluted with 0.02 mol/l HCl. The results are shown in Figs. 6-51 and 6-52. Samples of 1 ml were taken from fraction 26 (Fig. 6-51) and fractions 23, 25 and 27 (Fig. 6-52) and freeze-dried. They were re-dissolved in SDS sample buffer and separated by SDS electrophoresis as shown in Plate 6-20 and 6-21. Fractions 23, 25 and 27 (Fig. 6-52) ran as broad bands. Fraction 25 (Fig. 6-52) was similarly prepared and run on cationic electrophoresis (see Plate 6-17), and was found to be multi-banded indicating a heterogeneous sample which was quite different from that indicated by the SDS electrophoresis.

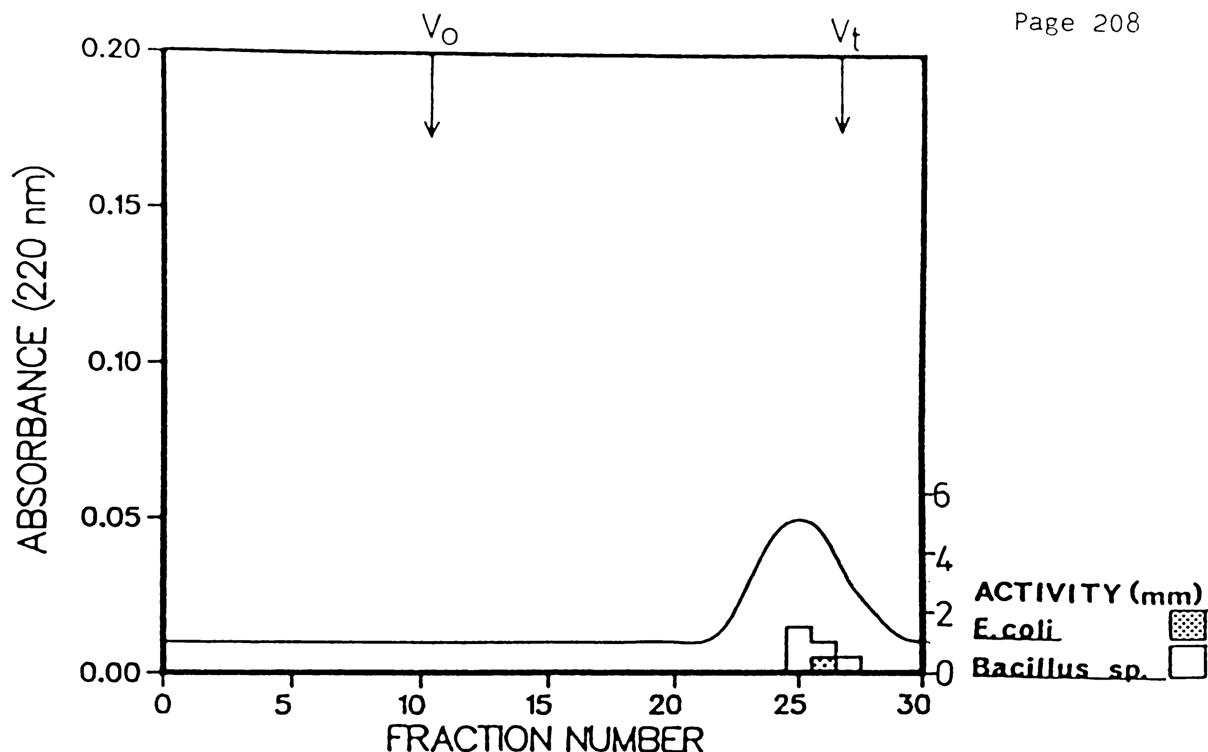


Fig. 6-51: Gel Filtration Chromatography of Fractions 15-20 from Fig. 6-50
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 15-20 (Fig. 6-50), urea removed, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

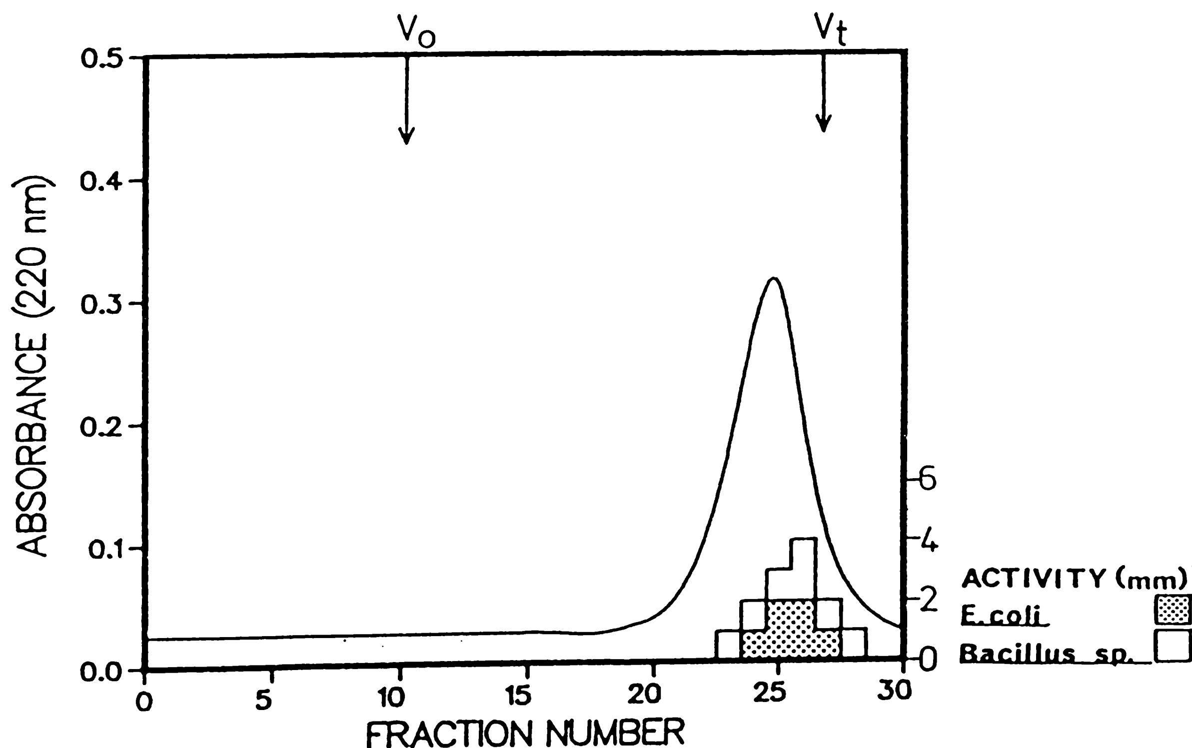


Fig. 6-52: Gel Filtration Chromatography of Fractions 21-25 from Fig. 6-50
Gel: Sephadex G 50 F
Eluent: 0.02 mol/l HCl, pH 1.7
Sample: Fractions 21-25 (Fig. 6-50), urea removed, evaporated to 5 ml and adjusted to pH 1.7
Fraction Volume: 10 ml

6.8 Conclusions

The sheep thymus preparation is a very complex mixture. The disaggregating procedures outlined using EDTA and urea were largely unsuccessful. Although single bands were often achieved on SDS electrophoresis, the same samples were multi-banded on cationic electrophoresis. The most likely reason for this is that there are a number of peptides present with similar migration rates on SDS electrophoresis, or that re-aggregation of the compound(s) occurrs in the non-dissociating cationic eletrophoresis system. The results obtained from the preparative electrophoresis are probably the most useful in trying to understand what antibacterial compounds are present in the thymus preparation. There appear to be two antibacterial compounds which have a slower migration rate than lysozyme. There were also 2 or 3 compounds which have a faster migration rate than lysozyme. Results from the separation procedure using 6 mol/l urea (Section 6.6) established the presence of a lysozyme-like protein with a migration rate on both cationic and SDS electrophoresis similar to that of egg white lysozyme. This lysozyme-like protein was not found during the separation procedures using preparative electrophoresis or after dialysis in EDTA. Yet certain compounds exhibited lytic activity but were shown to migrate differently on SDS and cationic electrophoresis to bovine lysozyme found by Eschenbruch (1980) and later isolated in section 6.6. For example, fraction 15 (Fig. 6-34) exhibited lytic activity, but on SDS electrophoresis it stained as a broad band with an estimated molecular weight of approximately 11 000. This unusual behaviour of lysozyme is difficult to understand but it seems to be a completely separate event from the inactivation seen in the original preparation. After gel filtration chromatography (Fig. 6-1) the higher

molecular weight fraction exhibited lytic activity. The inactivation of lysozyme is probably due to the presence of spermine and spermidine which is removed during the gel filtration chromatography. Grossowicz and Ariel (1963) reported that spermine and spermidine prevent lysozyme from lysing *Micrococcus lysodeikticus* by stabilising the cell membrane.

However, inactivation of lysozyme was detected which was not due to the presence of spermine or spermidine. In the separation shown in Fig. 6-38, for example, no lytic activity was detected in the fractions. However after further gel filtration chromatography lysozyme activity was detected and bovine lysozyme was subsequently isolated.

CHAPTER SEVEN

DISCUSSION

This study showed that spermine was not responsible for the antibacterial activity in bovine beta-lysin, seminalplasmin and the milk cell extract. However spermine was isolated from the calf thymus preparation. Eschenbruch (1980) had previously isolated the "thymus peptide" from this preparation and found that it behaved in an identical manner on electrophoresis to the bovine rumen peptide which Briggs (1983) subsequently identified as spermine. Therefore to discover that the "thymus peptide" was spermine was not surprising. However only part of the preparation Eschenbruch worked on was found to be spermine. A second "peptide" isolated from a lysozyme-containing fraction of higher molecular weight by ion exchange chromatography was found to contain a mixture of antibacterial cationic proteins and peptides and not spermine (although it was found to have a migration rate on cationic electrophoresis similar to that of the other "thymus peptide"). The difference between the two peptide extracts could be accounted for by the fact that the lysozyme-containing fractions were taken from fraction numbers 14-23 from a Sephadex G 25 gel filtration column while the "thymus peptide" was recovered from fractions 25-26. With such narrow separation, and also with regard to the fact that elution of a spermine standard from such a column is spread over several fractions (Briggs, 1983), it is highly likely that Eschenbruch (1980) obtained spermine that had co-chromatographed with, rather than was bound to the lysozyme -containing fraction. The spermine would then have been eluted from the ion exchange column with alkali. In addition, Briggs (1983) found that some cationic substances and spermine migrate similarly on cationic

electrophoresis. Therefore the cationic compounds present may have appeared to be similar to the other "thymus peptide" from the electrophoresis results.

In this study, however, the cationic peptides removed by alkali from the ion exchange column did not have migration rates similar to that of spermine (which travels near the front) on cationic electrophoresis. Also, acid hydrolysis did not reveal any spermine in this fraction. Acid hydrolysis was found to be a useful and reliable technique in the detection of spermine and spermidine. Bachrach (1976) showed that it was not possible to detect polyamines in plasma or urine unless the sample was subjected to acid hydrolysis.

Acid hydrolysis also confirmed the absence of spermine or spermidine from bovine beta-lysin. Beta-lysin was found to be a cationic peptide with a molecular weight estimated by SDS electrophoresis to be approximately 6 100. This was similar to the result obtained by Johnson and Donaldson (1968). However their preparation was found to be heterogeneous and was purified to homogeneity by gel filtration chromatography. Further work in the present study was not carried out on beta-lysin as Carroll and Martinez (1981a,b,c) published a characterisation of rabbit beta-lysin. In their work the preparation appeared homogeneous on column chromatography and analytical SDS polyacrylamide gel electrophoresis. Analysis of dansylated or ^{125}I-labelled samples in peptide-resolving polyacrylamide gels revealed a single band with a molecular weight of 1 800. They labelled this low molecular weight, heat-stable polypeptide fraction PC-III. Amino acid analysis of PC-III showed it was a single, 2,000 dalton peptide composed of 17 amino acid residues, 24% basic and 35% nonpolar (Carroll and Martinez, 1981b). Small amounts of carbohydrate

and lipid were also detected. However removal of these did not significantly alter the antibacterial activity.

The antibacterial activity in seminalplasmin was also found not to be due to spermine. It was found to be impossible to purify seminalplasmin according to the method of Reddy and Bhargava (1979) as seminalplasmin was lost through the dialysis tubing. Using an alternative purification method seminalplasmin was isolated and found to be a cationic peptide with a molecular weight estimated by SDS electrophoresis (using the method of Laemmli, 1970) to be 7 500. Subsequently Theil and Scheit (1983a) published a new method for the purification of seminalplasmin. They followed the procedure of Reddy and Bhargava (1979) but also found that the dialysis step led to excessive losses of seminalplasmin. This problem was circumvented by using benzoylated dialysis tubing. In addition the last step of the procedure involved preparative high performance liquid chromatography on a reverse phase column. The highly purified seminalplasmin was found on ultracentrifugation to have a molecular weight of 6 300 daltons. The amino acid sequence of seminalplasmin was also determined (Theil and Scheit, 1983b). The number of amino acids according to the sequence was 48, giving a calculated molecular weight of 6 385.

The molecular weights of the two antibacterial substances isolated from the milk cell extract of Hibbitt <u>et al</u>. (1971) were larger than this. The higher molecular weight protein was found to exhibit lytic activity which was not detected in the original preparation. It was also found to have an isoelectric point greater than pH 9.5, and is therefore similar in many respects to bovine lysozyme. The molecular weights estimated for these proteins from SDS electrophoresis were between 17 000-21 000 for the larger protein and 11 000 for the smaller

protein.

The different molecular weight values for the larger protein were obtained from two SDS electrophoresis systems at different pH values (pH 7 and 8.3). The difference may be explained in terms of the degree of SDS binding at the two pH values. At pH 7 the protein would be more cationic, thus having a lower net charge which would result in a slower migration rate: hence a higher molecular weight value would be calculated. Other workers have demonstrated that cationic proteins behave anomalously when submitted to electrophoresis in the presence of SDS (Waehneldt, 1975). Generally, the apparent molecular weights of basic proteins are too high. This was shown for ribonuclease and lysozyme by Shapiro et al. (1967), who also noted that deviations from the correct mobility are more pronounced in gels with a low polyacrylamide concentration. Similarly, histones and cytochrome c were shown to have apparently increased molecular weights when submitted to SDS electrophoresis (Panyim and Chalkley, 1971).

Lehtovaara (1978) gave three reasons why some proteins do not migrate according to their molecular weight:

(a) poor SDS binding to acidic proteins or protein containing rigid S-S bonds or carbohydrate,

(b) neutralisation of the negative charge of SDS by basic proteins,

(c) abnormal conformation of protein-SDS complexes.

The error in determining histone molecular weights has been attributed to a lower net negative charge on the SDS complex due to a high positive charge on the histone chains (Noelken et al., 1981; Panyim and Chalkley, 1971; Swank and Munkries, 1971). Hamana (1981) on the other hand concluded that anomalous behaviour of histones in SDS

polyacrylamide gel electrophoresis is primarily due to the effective size of SDS-histone complexes rather than the neutralisation of the negative SDS charge. Therefore, values for the molecular weight for cationic antibacterial proteins by SDS electrophoresis must be viewed cautiously. The molecular weight value obtained for bovine beta-lysin (6 100) by SDS electrophoresis was considerably higher than the value obtained for PC-III (1 800) by Carroll and Martinez (1981a). This discrepancy may be explained by the different SDS electrophoresis methods used. Carroll and Martinez used a variation of the method of Swank and Munkries (1971), where the use of 1% SDS with 8 mol/l urea reduces the gel porosity, giving improved separation of peptides. The molecular weight value obtained using this method correlated very well with the calculated molecular weight value from amino acid analysis (2 000).

Unusual SDS electrophoresis results were also obtained during the study of the sheep thymus preparation. Broad bands were often obtained which were not a result of sample overloading. These broad bands may be a result of variable SDS coating of the protein or of there being several substances with slightly different mobilities. In many instances, when these samples were separated on cationic electrophoresis multiple bands were obtained. Again it is not certain whether this is representing several substances or merely the formation of aggregates in the non-denaturing system. In the preparative electrophoresis (Section 6.5) broads bands on SDS electrophoresis in some instances gave single bands on cationic electrophoresis.

The molecular weight value obtained for seminalplasmin on SDS electrophoresis (7 500) was close to the calculated value of 6 385) calculated from amino acid sequence. The 1 000 dalton difference can be explained considering its cationic nature and low molecular weight. Ideally, results obtained from SDS electrophoresis should be confirmed by amino acid analysis. However analytical ultracentrifugation appears to be a useful technique for molecular weight estimations as the result obtain for seminalplasmin (6 300) correlated exceptionally well with the calculated value (6 385).

Hamana (1981) also obtained the correct values of molecular weight for histones by gel electrophoresis in the presence of the cationic surfactant cetyl trimethylammonium bromide (CTAB). The correlation between the retardation coefficient and molecular weight for CTAB-histone complexes was linear. The retardation coefficient is a measure of the effective surface area of a particle independent of different positive charges (Hamana and Iwai, 1974).

Anomalies are found also when using the technique of isoelectric focusing for the estimation of the isoelectric points of cationic peptides and proteins. With the bovine beta-lysin and seminalplasmin samples, no bands were detected after staining the isoelectric focusing gel. This may be due to the molecular size of these peptides. Righetti and Chillemi (1978) reported that peptides below a molecular weight of 10 000 are increasingly difficult to fix and stain. Other difficulties in analysis of cationic proteins by isoelectric focusing are cathodal drift, uneven electrical conductance of focused ampholines and interference by carbon dioxide from the atmosphere (Delinceé and Radola, 1978; Thomas and Hodes, 1978). Carbon dioxide interferes with the determination of pI values above 8.2 and for this reason Delinceé and

Radola (1978) recommended that isoelectric focusing be carried out in a carbon dioxide-free atmosphere. Valkonen and Piha (1980) recorded pI values for cytochrome c measured in the absence and presence of carbon dioxide, as 10.25 and 9.25 respectively.

In the investigation of the milk cell extract, isoelectric focusing was employed in an attempt to separate the components, but a large degree of streaking was obtained. It was observed that during the focusing components were disaggregating from each other and refocusing, and even after four hours streaking was still apparent on the stained gel. When long runs of up to 18 hours were conducted in a carbon dioxide-free atmosphere, in an attempt to reduce the streaking, severe distortions of the gel resulted. Valkonen and Piha (1980) also found prolonged focusing gives pH drift and decay of the pH gradient. Although 6 mol/l urea is often used to prevent reaggregation and to give good resolution and reproducibility, this modification introduces many problems. Artefacts may be caused by carbamylation of amino groups by cyanate produced by decomposition of urea. Goldsmith et al. (1979) suggested that freshly prepared or ion exchange-treated urea should be used. Also focusing time should be as short as possible to prevent further decomposition of urea, as the risk of carbamylation is greatest in the alkaline region (Stark et al., 1960). In addition, urea affects the isoelectric points of proteins (Josephson et al. 1971), although a correction can be made (Thomas and Hodes, 1978).

Satisfactory focusing was obtained with the lysozyme-like proteins isolated from sheep thymus, seminal plasma and the milk cell extract. The proteins were focused in bands along the cathode wick, indicating isoelectric points greater than pH 9.5. In order to obtain a more accurate value for these proteins a higher pH gradient would be

necessary. Valkonen and Piha (1980) described an isoelectric focusing technique which enabled bovine liver histones to be separated and their isoelectric points recorded. This method used a narrow pH gradient (9.0-11.0) and was carried out in an atmosphere of nitrogen. During this study narrow pH gradients from 9-11 were not used. In previous attempts with these gels pH gradients higher than 10 have rarely been achieved. However, as mentioned earlier, the absence of carbon dioxide is critical. Extension of pH gradients to above 11 has been achieved by the addition of N,N,N',N'-tetramethylethylenediamine to polymerising gels made with ampholytes which have a pH range of 3.5-9.5 (Yoa-Jun and Bishop, 1982).

Apparent heterogeneity on isoelectric focusing is another problem. The lower molecular weight protein isolated from the milk cell extract gave a single band with SDS electrophoresis but appeared heterogeneous on isoelectric focusing. This protein may be heterogeneous, but other factors during isoelectric focusing could be responsible. Apparent heterogeneity can be caused by instability of a molecule at its pI, conformational transition, interaction with carrier ampholytes, metal chelation, electrode modification, denaturation or precipitation (Righetti and Drysdale, 1976). Righetti _et al_. (1979) showed that microheterogeneity was a common status of proteins and that homogeneous proteins only exist for a short time at the end of a purification process or when the purified sample is stored in liquid nitrogen. They suggested that the heterogeneity was due to deamidation which is a spontaneous chemical process.

Recently, Bjellqvist et al. (1982) described an isoelectric focusing technique in immobilised pH gradients. The pH gradient gels are cast in the same way as pore gradient gels, but instead of varying the acrylamide content, the light and heavy solutions are adjusted to different pH values with the aid of Immobiline buffers. The buffering groups are covalently linked to the matrix. There are a number of advantages for the Immobiline gel:

- true equilibrium, no drift
- higher resolution
- higher loading capacity
- better control of form, width, ionic strength and buffering capacity
- possibility of generating extremely shallow pH gradients
- easier separation of buffering species from proteins in preparative runs
- insensitivity to salts and buffers in the sample.

In addition, peptide analysis is possible as the peptide zones can be revealed by any stain for primary and secondary amino groups (e.g. ninhydrin, fluorescamine, dansylchloride) unlike with conventional ampholytes (Gianazza et al., 1983). Isoelectric focusing in immobilised pH gradients may well allow the accurate determination of the isoelectric points of cationic peptides.

Difficulties were also encountered in other electrophoretic studies. The electrophoretic analysis of cationic substances at pH 4.5 (Reisfeld et al., 1962) was often unsuitable as at this pH they may all have a high positive charge and therefore on electrophoresis are all very mobile and poorly separated. During the purification of bovine beta-lysin, the mobility of spermine and beta-lysin were identical on cationic electrophoresis at pH 4.5. Similarly, Briggs (1982) found some

cationic compounds migrated identically at pH 4.5, but quite differently using cellulose acetate electrophoresis at pH 8.6-9.0. These problems may be solved using a new discontinuous buffer system for cationic proteins, carried out at pH 6.8 (Thomas and Hodes, 1981).

Another problem encountered during cationic electrophoresis was the possible aggregation that was found with the sheep thymus proteins. Apparently single bands on SDS electrophoresis gave multiple bands on cationic electrophoresis. Peterson (1972) suggested that a protein need not be heterogeneous if it shows more than one band on electrophoresis. Artefacts can be produced by partial reaggregation into higher molecular weight polymers. Artefacts may also be produced by protein-buffer interactions in gel electrophoresis.

The dissociation of complexes into monomers of the protein may be achieved by the use of urea. Bonner et al. (1968) modified Reisfeld's method for the electrophoresis of histones by the addition of 6 mol/l urea to the 15% separating gel. The use of pure urea is essential because of the decomposition to produce cyanate as mentioned earlier.

Charge heterogeneity can also cause artefacts (O'Farrell, 1975). Modification of amino groups in proteins may occur even in mild conditions. Asparagine and glutamine are known to undergo spontaneous deamidation to give products with different isoelectric points.

Similarly, many irregularities also occurred using other biochemical separation techniques. Gel filtration chromatography was used extensively in order to purify the antibacterial compounds. There is a linear relationship between the logarithm of the molecular weight of a protein and its elution volume. However, many anomalies result from gel-protein interaction. Substances appearing later are retarded

by adsorption or by electrostatic interaction whereas early elution is caused by ion exclusion or by complex formation or aggregation (Janson, 1967). Electrostatic interactions, including ion exclusion, are due to cross-linked dextran chains containing a few terminal carboxylic acid groups: negatively charged substances are excluded from the gel phase. Small amounts of cations will be adsorbed with the more tightly cross-linked Sephadex G 50 to G 10 gels and at low ionic strength the effect is more noticeable. If changes in the ionic strength of the eluent result in changes of solute behaviour, then ionic interactions between solute molecules and the gel filtration medium may be suspected. Peptides and polypeptides with molecular weights up to about 5 000 do not show the same regularity on gel filtration in dilute aqueous buffer solution as do the globular proteins (Andrews, 1970).

Examples of gel-protein interaction occurred repeatedly during this study. Gel filtration chromatography of similar beta-lysin samples on Sephadex G 25 resulted in different elution patterns. In the first instance the beta-lysin was eluted near the bed volume of the gel, and later when repeating the procedure the beta-lysin was eluted in the void volume. The molecular weight of beta-lysin was estimated to be approximately 6 100. Therefore gel-protein interactions were probably responsible for beta-lysin being eluted near the bed volume of the gel. Also, during the purification of the milk cell extract and the sheep thymus preparation differences in elution volumes of the same substance were noticed, especially when different eluents were used. Distilled water was often used as an eluent to utilise the gel-protein interactions in order to obtain separation. Large changes in the elution volume occurred, with the sample being eluted near the bed volume of the gel.

Aggregation during gel filtration chromatography was also apparent in the purification of the milk cell antibacterial proteins. In the initial gel filtration step with EDTA in the eluent the antibacterial compounds were spread throughout the elution profile. Aggregation was also apparent in the next step in which the fractions of lower molecular weight from the gel filtration step with EDTA were re-chromatographed on Sephadex G 50 to give active peaks at the void volume and bed volume.

Aggregation was also apparent from electrophoresis results in the purification of the sheep thymus preparation. The molecular weights that were found on SDS electrophoresis were larger than those estimated for the same samples from the elution volume from the gel filtration column. Also in many instances a single band on SDS electrophoresis gave multiple bands on cationic electrophoresis at pH 4.5.

Lysozyme also displayed unusual behaviour during the purification of the sheep thymus extract. Lysozyme was eventually isolated from the preparation using 6 mol/l urea, and was shown to have a migration rate on both cationic and SDS electrophoresis similar to egg white lysozyme. However earlier attempts to isolate lysozyme yielded broad bands on SDS electrophoresis with molecular weights around 11 000. These compounds exhibited lytic activity, so if they were actually lysozyme the binding substance(s) would be neutralising the cationic charges on lysozyme and not disrupting its tertiary structure as it is still lytically active. However, lysozyme was found to be initially inactive in the sheep thymus preparation. Eschenbruch (1980) suggested that the calf thymus preparation contains an inhibitor as it could be shown that the whole preparation inhibits the activity of egg white lysozyme. Inhibition is probably related to the presence of spermine and spermidine: when these were removed from the preparation lytic activity was observed. However,

Grossowicz and Ariel (1963) showed that lysozyme activity (hexosamine liberation) was actually increased in the presence of spermine and that lysozyme did not bind with spermine. In the presence of spermine, pre-grown cells are actually lysed by lysozyme (hexosamine released) but clearing is not observed as the polyamine binds to the cell membrane and stabilised protoplasts remain. Eschenbruch (1980) did not discriminate between inhibition of lytic activity and the possible stabilisation of the protoplasts.

Inhibition of lysozyme activity has been shown to result from cationic substances binding to the lysozyme anionic catalytic sites. Hayashi et al. (1968) showed that cationic detergents interact with the active site of lysozyme, causing inhibition. Modification of lysozyme action by cationic substances was also described by Ginsburg and Sela (1976). They showed that both lysine- and arginine-rich histones, and protamine sulphate, as well as cationic proteins which were obtained from human blood leukocytes by the of Zeya and Spitznagel (1968) prevented to a large extent the lysis of Micrococcus lysodeikticus by lysozyme. As the inhibition of lysis was stronger when lysozyme was pre-incubated with the cationic substances, inactivation of the lysozyme action rather than protection of microorganisms from the lytic action of lysozyme by binding of the cationic components to the bacterial cell surface was suggested. In the current work the lysozyme-like protein isolated from the milk cell extract was also inhibited in the original preparation. As spermine or spermidine was not isolated from this preparation, presumably some other inhibitor was involved.

The lytic activity in the milk cell extract may not have been apparent due to low concentration and activity of the lysozyme-like protein. Lysozyme from different sources may vary in degree of activity. Eschenbruch (1980) found that bovine lysozyme has a lower specific activity than egg white lysozyme. Chandan et al. (1965) found similar results for bovine milk lysozyme. Similar observations were made by Carroll and Martinez (1979) when they compared rabbit lysozyme with human lysozyme: not only was the lysozyme concentration much lower in rabbit serum, but the enzyme itself was also much less bacteriolytically active. Additionally, bacteria sensitive to egg white lysozyme are not always sensitive to lysozymes from other sources. This was shown by Vakil et al. (1969) when comparing the sensitivity of some Gram-negative and Gram-positive bacteria with lysozymes derived from bovine milk, human milk and hen egg white. Thus, bovine milk lysozyme lysed Micrococcus lysodeikticus, Sarcina lutea and Bacillus subtilis. Human milk lysozyme was bacteriolytic for Micrococcus lysodeikticus, Sarcina lutea and Streptococcus lactis. Neither milk lysozyme was active against any of the Gram-negative bacteria tested. Egg white lysozyme lysed all these microorganisms and in addition - if to a lesser extent - Staphylococcus aureus, Streptococcus faecalis and Gram-negative Pseudomonas fluorescens. When NaCl or EDTA, or a combination of these, were included in the assay, differences in susceptibility to the various lysozymes became even more pronounced.

The dissolution of pre-grown Micrococcus lysodeikticus cell walls should not be the sole property used in classifying an enzyme as a lysozyme enzyme, as cell lysis can be brought about by proteolytic enzymes (Salton, 1957).

The characteristics of lysozyme according to Jollès _et al_. (1974) are:

(1) a basic protein

(2) lyses _Micrococcus lysodeikticus_ cells

(3) of low molecular weight (approximately 15 000)

(4) stable to heating, particularly under acidic conditions

(5) labile at alkaline pH values

(6) action on appropriate substrate liberates compounds which can be detected by reagents for reducing sugars or amino sugars.

All the lytic proteins isolated in this study were shown to be cationic as they focused at the cathodic end of the isoelectric focusing gel which indicated isoelectric points greater than 9.5. They were also shown to lyse _Micrococcus lysodeikticus_ cells on agarose plates and to have low molecular weights, except the lysozyme-like protein from the milk cell extract (molecular weight 17 000-21 000).

Although the lytic proteins have many similar characteristics to lysozyme, they cannot be called lysozyme at this stage. According to Strominger and Ghuysen (1967), "The term lysozyme should be restricted to that group of basic proteins which are thermostable and which catalyse the hydrolysis of the glycosidic linkage between acetylmuramic acid and acetylglucosamine." It would therefore be necessary to determine the nature of the lytic action.

Studies concerning the heat stability showed that the activity of the antibacterial proteins were unaffected by heating at neutral pH. The milk cell extract was boiled for 10 minutes without any noticeable loss of activity although a heavy precipitate formed. The temperature stability of the lytic activity was unable to be checked owing to its inhibition in the crude preparation. Unfortunately the thermal denaturation of the inactive protein did not help in the further separation of the antibacterial compounds. Similarly, beta-lysin is reported to be heat-stable, losing little activity after incubation at 95°C for 30 minutes (Johnson and Donaldson, 1968). Seminalplasmin is also heat-stable being relatively unaffected by heating at 90°C for 10 minutes. Hirsch (1956a) found that phagocytin withstood heating to 65°C for several hours. Skarnes and Watson (1956a) reported that the activity of an antibacterial thymus peptide was not destroyed by boiling for up to one hour. Similarly, heat stability has also been demonstrated for cationic proteins derived from leukocytes (Odeberg and Olsson, 1975); Weiss _et al_., 1975); Walton, 1978). It appears to be a common property of these cationic proteins with relatively low molecular weight. The exceptional heat stability may be due to the absence of a tertiary structure.

Another factor that has to be considered with these low molecular weight proteins is the possibility that they are artefacts produced as a result of the method of extraction. Degradation of proteins by proteinases could occur. Crampton _et al_. (1957) found that changes in the methods of preparation of histones gave rise to products with different chemical properties and that enzymic cleavage appeared to be the primary cause of such changes. Furlan and Jericijo (1967) also demonstrated autolytic breakdown of nucleoproteins by proteases present

in calf thymus nuclei. Incubation of isolated nuclei or deoxyribonucleic protein generated new components by proteolysis at the expense of authentic histones. These products had similar properties to those of histones (Stellwagen, 1968).

Recently, Hannappel et al. (1982) showed that the peptide thymosin $\alpha 1$ isolated from calf thymus by Low et al. (1979), which has a molecular weight of 3 350, is absent or present in only trace amounts when using a procedure that minimises the possibility of proteolytic modification.

Extraction methods which are carried out at a very low pH may cause acid hydrolysis of certain proteins, and the antibacterial peptides may be a product of hydrolysis rather than a natural tissue constituent. Therefore, methods such as those used by Dubos and Hirsch (1954) should be avoided and extraction procedures should be carried out in the cold to keep denaturation to a minimum.

However, many workers have succeeded in isolating antibacterial cationic proteins using milder methods. Reddy and Bhargava (1979) isolated seminalplasmin from bovine seminal plasma by ion exchange and gel filtration chromatography at pH 7.4. Beta-lysin was also isolated at near neutral pH (Johnson and Donaldson, 1968). Similarly, Olsson and Venge (1974) extracted antibacterial proteins of low molecular weight with sodium acetate buffer at pH 4 and 0°C. Walton (1978) used acetate buffer at pH 4 for the extraction of cationic proteins from rabbit polymorphonuclear leukocytes. These methods are no more harsh than other methods employed for the isolation of biological materials, and are definitely milder then those employed for the extraction of histones.

The reproducibility of the extraction methods was also a problem. With the milk cell extract the heat stability results were different from those obtained by the original authors. Also the sheep thymus preparation and purification procedure as outlined by Eschenbruch (1980) gave different results. In the course of the investigation the extraction procedures were carried out several times, and the resulting material appeared to be the same in each case. As the procedures involved several steps of dialysis, heating and fractionation with acetone and ethanol (for the sheep thymus preparation), it is possible that the original authors isolated a preparation that was different from that used in this investigation. The use of different tissues (sheep thymus in this study, calf thymus in the investigation of Eschenbruch) may also account for some of the differences in results. Reproducibility of the extraction of seminalplasmin from bovine seminal plasma was also unreliable owing to the dialysis steps which allowed the loss of the seminalplasmin. The reproducibility of results can also be altered by using different microorganisms in the antibacterial assays, which have different susceptibilities to the various antimicrobial compounds. The detection of lysozyme during the purification of seminalplasmin is a good example.

Without knowledge of the exact intracellular concentrations of spermine in tissues and secretions it is not possible to make any definite suggestion as to the likely role of spermine. Bovine tissues contain amine oxidases and if present in an unbound form the polyamine would give rise to oxidation products which are known to be toxic to bacteria and viruses. Bachrach and Leibovic (1966) showed that oxidised spermine can penetrate viruses and combine with nucleotides or DNA and impair biological activity. Therefore if under certain conditions

oxidation products are formed, spermine may have an antimicrobial role *in vivo*. To further support the role of polyamines as antibacterial agents, Kido *et al*. (1980) isolated a new antibiotic (B-1008) from the cultured broth of *Pseudomonas fluorescens*. This basic substance contains a spermidine moiety and possesses antibacterial activity against a wide range of bacterial species. There are other antibiotics containing spermidine such as LL-BM-123 (Ellestad *et al*., 1978), edeines (Hettinger and Craig, 1970), bleomycin A_5 (Fujii *et al*., 1973), and laterosporamine (Shoji *et al*., 1976).

Recently, specific extracellular polyamine-binding proteins have been described in rabbit and human serum (Bartos *et al*., 1980; Roch *et al*., 1979), and covalent complexes have been described in human amniotic fluid (Chan *et al*., 1979). Mezzetti *et al*. (1980) reported that chick duodenal mucosa contains a cytoplasmic protein capable of binding selectively to spermine with high affinity. More recently Mezzetti *et al*. (1982) found that human peripheral lymphocytes contain a cytosolic factor able to bind spermine non-covalently and with high affinity. The factor is heat sensitive and appears to be a protein which is an intracellular, specific binder for spermine. In physiologically growing tissues such as duodenal mucosa of developing chick embryo it has been found that polyamine-binding proteins respond to the growth stimulus by rapidly modifying their activity. Further work may show conclusively that polyamines have essential functions in cellular metabolism.

The opposite conclusion, however, may be drawn from observation that spermine inhibits the immune response. Curry *et al*. (1980), while working on certain naturally occurring, purportedly macromolecular inhibitors of lymphocyte transformation identified the inhibitory agent as spermine. The spermine bound to, and was eluted with, the protein.

Also, Byrd *et al.* (1977, 1978) showed that a variety of *in vitro* parameters of immunity are inhibited by polyamines added to cultures containing bovine serum.

In this study of the sheep thymus preparation spermine and spermidine did not appear to form spermine-protein complexes. The polyamines were separated from the major part of the protein material during gel filtration chromatography using mild conditions (0.1 mol/l Tris buffer, pH 7.4). A small amount of peptide material was eluted with the polyamines but this was removed by further ion exchange chromatography. The role of free spermine in the thymus preparation is unclear. Its antibacterial properties *in vivo* may be of importance, however in view of the findings discussed above this may only be a secondary role or it is probably not involved at all.

The alternative possibility that the antibacterial activity in the various mammalian tissues and secretions may be due to a single small molecule other than spermine (a peptide) seems unlikely. The existence of several well characterised cationic proteins in seminal plasma, serum, and polymorphonuclear leukocytes is well established. For example, seminalplasmin (Reddy and Bhargava, 1979; Theil and Scheit, 1983a,b), PC-III (Carroll and Martinez, 1981a,b,c), bactericidal/permeability-increasing protein, BPI (Elsbach and Weiss, 1983) and MCP-1 and MCP-2 (Lehrer *et al.*, 1983) are all proteins that have been unequivocally purified to homogeneity and are not composed of subunits or aggregates. However, these compounds may exist *in vivo* as aggregates and may be responsible for the activity of the less purified antibacterial compounds which have been reported.

The results obtained in this study do not support the possibility of a single small cationic compound being responsible for the antibacterial activity in the various mammalian tissues and secretion that were examined. The cationic proteins that were isolated from beta-lysin, seminal plasma and the milk cell extract were all different from each other as shown by their behaviour on ion exchange chromatography, SDS and cationic electrophoresis. Some similarity was seen between the lytic proteins which were isolated.

In mammalian tissues, the cationic peptides and proteins contribute to the overall primary non-specific defence mechanism. Hirsch and Dubos (1954) discussed the possibility that these antibacterial peptides, which under normal conditions may be associated with acidic molecules, are released as the result of autolytic processes that accompany inflammation and infectious necrosis, and thus exercise some control of pathogens. Results of Bloom et al. (1947) also indicate the possible importance in vivo of the strongly cationic polypeptide they isolated from calf thymus: when injected into mice, it protected them against experimental infections with Bacillus anthracis.

Recently, a antibacterial substance of low molecular weight has been isolated from rabbit peritoneal exudate (Scales and Kluger, 1984). This antibacterial substance has a molecular weight less than 5 000 daltons and heat treatment (70°C, 1 hour) had no influence on its activity. Interestingly this factor was more active at 41°C (febrile temperature for rabbits) than at 39°C (afebrile temperature). Temperatures simulating fever seem to have a beneficial influence upon several host resistance mechanisms. The increased antibacterial activity of this substance at febrile temperatures suggests it is of importance in vivo.

According to Elsbach et al. (1979), the catalytically effective proteins of the polymorphonuclear leukocytes, i.e. lysozyme, myeloperoxidase, and some proteases, have a limited bactericidal potency by themselves, whereas the cationic proteins are strongly antibacterial and may therefore contribute significantly to the killing of ingested microorganisms. The observations of these workers suggest that the Bactericidal/permeability-increasing protein (BPI) is the principal bactericidal agent in the intact neutrophil against E. coli and S. typhimurium (Elsbach and Weiss, 1983). However, it is difficult to establish the relative importance of any of the microbicidal systems of the leukocytes. Only if the decreased host resistance to infection can be directly attributed to the deficiency of a single component can conclusions be drawn as to its significance in vivo.

Further studies of these cationic compounds is needed to fully understand their role in the non-specific immune response.

Abe, K. and Samejima, K. (1975) Anal. Biochem. 67, 298-308.

Abraham, A.K., Olsnes, S. & Pihl, A. (1979) Fidelity of protein systhesis in vitro is increased in the presence of spermidine. Federation of European Biochemical Societies Letters, 101, 93-96.

Abraham, A.K. & Pihl, A. (1980) Variable rate of polypeptide chain elongation in vitro. European Journal of Biochemistry, 106, 257-262.

Abraham, A.K. & Pihl, A. (1981) Role of polyamines in macromolecular sythesis. Trends in Biochemical Sciences, 6, 106-107.

Andrews, P. (1970) Estimation of molecular size and molecular weights of biological compounds by gel filtration chromatography. In: Methods of Biochemical Analysis Eds. Glick, D., Vol 18, 1-53, Interscience N.Y., London.

Atkins, J.F., Lewis, J.B., Anderson, C.W. & Gesteland, R. (1975) Enhanced differential synthesis of proteins in a mammalian cell-free system by addition of polyamines. Journal of Biological Chemistry, 250, 5677-5695.

Bachrach, U. (1973) Functions of Naturally Occurring Polyamines, Academic Press, New York.

Bachrach, U. (1976) Polyamines as chemical markers of malignancy. Italian Journal of Biochemistry, 25, 77-93.

Bachrach, U. & Leibovici, J. (1966) Inhibitory effect of oxidised spermine on the multiplication of coliphage T5. Journal of Molecular Biology, 19, 120-132.

Bartos, D., Bartos, F., Campbell, R.A., Grettie, D.P. & Smejtek, P. (1980) Antibody to spermine: A natural biological constituent. Science, 208, 1178-1181.

Bartos, F. & Bartos, D. (1978) Antipolyamine antibodies. Advances in Polyamine Research, 2, 65-70.

Beckerdite, S., Mooney, C., Weiss, J., Franson, R.C. & Elsbach, P. (1974) Early and discrete changes in permeability of Escherichia coli and certain other Gram negative bacteria during killing by granulocytes. Journal of Expermental Medicine, 140, 396-409.

Bjellqvist, B., Ek, K., Righetti, P.G., Gianazza, E., Gorg, A., Westermeier, R. & Poste, W. (1982) Isoelectric focusing in immobilised pH gradients: principle, methodology and some applications. Journal of Biochemical and Biophysical Methods, 6, 317-339.

Bloom, W.L., Watson, D.W., Cromartie, W.J. & Freed, M. (1947) Preparation and characterization of an anthracidal substance from various animal tissues. Journal of Infectious Diseases, 80, 41-52.

REFERENCES

Abraham, A.K., Olsnes, S. & Pihl, A. (1979) Fidelity of protein systhesis *in vitro* is increased in the presence of spermidine. *Federation of European Biochemical Societies Letters*, 101, 93-96.

Abraham, A.K. & Pihl, A. (1980) Variable rate of polypeptide chain elongation *in vitro*. *European Journal of Biochemistry*, 106, 257-262.

Abraham, A.K. & Pihl, A. (1981) Role of polyamines in macromolecular sythesis. *Trends in Biochemical Sciences*, 6, 106-107.

Andrews, P. (1970) Estimation of molecular size and molecular weights of biological compounds by gel filtration chromatography. *In*: Methods of Biochemical Analysis Eds. Glick, D., Vol 18, 1-53, Interscience N.Y., London.

Atkins, J.F., Lewis, J.B., Anderson, C.W. & Gesteland, R. (1975) Enhanced differential synthesis of proteins in a mammalian cell-free system by addition of polyamines. *Journal of Biological Chemistry*, 250, 5677-5695.

Bachrach, U. (1973) Functions of Naturally Occurring Polyamines, Academic Press, New York.

Bachrach, U. (1976) Polyamines as chemical markers of malignancy. *Italian Journal of Biochemistry*, 25, 77-93.

Bachrach, U. & Leibovici, J. (1966) Inhibitory effect of oxidised spermine on the multiplication of coliphage T5. *Journal of Molecular Biology*, 19, 120-132.

Bartos, D., Bartos, F., Campbell, R.A., Grettie, D.P. & Smejtek, P. (1980) Antibody to spermine: A natural biological constituent. *Science*, 208, 1178-1181.

Bartos, F. & Bartos, D. (1978) Antipolyamine antibodies. *Advances in Polyamine Research*, 2, 65-70.

Beckerdite, S., Mooney, C., Weiss, J., Franson, R.C. & Elsbach, P. (1974) Early and discrete changes in permeability of *Escherichia coli* and certain other Gram negative bacteria during killing by granulocytes. *Journal of Expermental Medicine*, 140, 396-409.

Bjellqvist, B., Ek, K., Righetti, P.G., Gianazza, E., Gorg, A., Westermeier, R. & Poste, W. (1982) Isoelectric focusing in immobilised pH gradients: principle, methodology and some applications. *Journal of Biochemical and Biophysical Methods*, 6, 317-339.

Bloom, W.L., Watson, D.W., Cromartie, W.J. & Freed, M. (1947) Preparation and characterization of an anthracidal substance from various animal tissues. *Journal of Infectious Diseases*, 80, 41-52.

Bonner, J., Chalkley, G.R., Dahmus, M., Fambrough, D., Fujimura, K., Ohlenbusch, H., Olivera, B. & Widholm, J. (1968) Isolation and characterization of chromosomal nucleoproteins. In: Methods of Enzymology. Eds. Grossman, L. & Moldave, K., Vol XIIB, 3-65, Academic Press, New York.

Bragg, P.D. & Polglase, W.J. (1963) Action of dihydrostreptomycin and antagonism by cations. Journal of Bacteriology, 85, 590-594.

Briggs, L.R. (1982) Antibacterial complexes of spermine and cationic proteins from bovine rumen. M. Phil. Thesis, University of Waikato, N.Z.

Brownlie, J. & Hibbitt, K.G. (1972) Antimicrobial proteins isolated from bovine cervical mucus. Journal of Reproduction and Fertility, 29, 337-347.

Brownlie, J., Howard, C.J. & Gourlay, R.N. (1974) Mucoplasmacidal activity of bovine milk for T.-mycoplasmas. Journal of Hygiene,Cambridge, 73, 415-423.

Byrd, W.J., Jacobs, D.M. & Amoss, M.S. (1977) Synthetic polyamines added to cultures containing bovine sera reversibly inhibit in vitro parameters of immunity. Nature, 267, 621-623.

Byrd, W.J., Jacobs, D.M. & Amoss, M.S. (1978) Influence of synthetic polyamines on in vitro responses of immunocompetent cells. Advances in Polyamine Research, 2, 71-83.

Carroll, S.F. & Martinez, R.J. (1979) Purification and properties of rabbit alvelolar macrophage lysozyme. Infection and Immunity, 24, 460-467.

Carroll, S.F. & Martinez, R.J. (1981a) Antibacterial peptide from normal rabbit serum. 1. Isolation from whole serum, activity, and microbicidal spectrum. Biochemistry, 20, 5973-5981.

Carroll, S.F. & Martinez, R.J. (1981b) Antibacterial peptide from normal rabbit serum. 2. Compositional microanalysis. Biochemistry, 20, 5981-5987.

Carroll, S.F. & Martinez, R.J. (1981c) Antibacterial peptide from normal rabbit serum. 3. Inhibition of microbial electron transport. Biochemistry, 20, 5988-5994.

Chan, W.Y., Seale, T.W., Shukla, J.B. & Rennert, C.M. (1979) Polyamine conjugates and total polyamine concentrations in human amniotic fluid. Clinica Chimica Acta, 91, 233-241.

Chandan, R.C., Parry Jr, R.M. & Shanani, K.M. (1965) Purification and some properties of human milk lysozymes. Biocimica et Biophysica Acta, 110, 389-398.

Cleland, W.W. (1964) Dithiothreitol, a new protective reagent for SH groups. Biochemistry, 3, 480-482.

Cohen, S.S. (1971) Introduction to the Polyamines, Prentice-Hall, Englewood, Englewood Cliffs, New Jersey.

Cohen, S.S. (1978) The functions of the polyamines. Advances in Polyamine Research, 1, 1-10.

Colom, J. & Subirana, J.A. (1979) Protamines and related proteins from spermatozoa of molluscs. Characterisation and molecular weight determination by gel electrophoresis. Biochimica et Biophysica Acta, 581, 217-227.

Coonrod, J.D. & Yoneda, K. (1983) Detection and partial characterization of antibacterial factor(s) in alveolar lining material of rats. The Journal of Clinical Investigation, 71, 129-141.

Crampton, C.F., Moore, S. & Stein, W.H. (1955) Chromatographic fractionation of calf thymus histone. Journal of Biological Chemistry,215, 787-801.

Crampton, C.F., Stein, W.H. & Moore, S. (1957) Comparative studies on chromatographically purified histones. Journal of Biological Chemistry, 225, 363-368.

Curry, M.C., Hussain, J.I., Smith, C.J. & Allen, J.C. (1980) Identification of a macromolecular inhibitor of in vitro lymophocyte proliferation in human seminal plasma as bound spermine. European Journal of Cell Biology, 21, 180-182.

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. & Jones, K.M. (1969) Data for Biochemical Research, Second Edition, Clarendon Press, Oxford.

Delincee, H. & Radola, B.J. (1978) Determination of isoelectric points in thin-layer isoelectric focusing. Importance of attaining steady-state and role of CO_2 interference. Analytical Biochemistry, 90, 609-623.

Donaldson, D.M. & Marcus, S. (1958) Studies on serum bactericidal activity. Interrelationships of heparin, citrate, protamine and γ-irradiation on serum and plasma bactericidal activity

Donaldson, D.M., Ellsworth, B., and Matheson, A. (1964). Separation and purification of beta-lysin from normal serum. J. Immunol. 92: 896-900.

Bacteriological Reviews, 41, [illegible]

Dubos, R.J. & Hirsch, J.G. (1954) The antimycobacterial activity of a peptide preparation derived from calf thymus. Journal of Expermental Medicine, 99, 53-63.

Ellestad, G.A., Cosulich, D.B., Broschard, R.W., Martin, J.H., Kunstman, M.P., Marton, G.O., Lancaster, J.E., Fulmor, W. & Lovell, F.M. (1978) Glycocinnamoylspermidine, a new class of antibiotics. 3. The structure of LL-BM123β γ_1, and γ_2. Journal of the American Chemical Society, 100, 2515-2524.

Cohen, S.S. (1971) Introduction to the Polyamines, Prentice-Hall, Englewood, Englewood Cliffs, New Jersey.

Cohen, S.S. (1978) The functions of the polyamines. Advances in Polyamine Research, 1, 1-10.

Colom, J. & Subirana, J.A. (1979) Protamines and related proteins from spermatozoa of molluscs. Characterisation and molecular weight determination by gel electrophoresis. Biochimica et Biophysica Acta, 581, 217-227.

Coonrod, J.D. & Yoneda, K. (1983) Detection and partial characterization of antibacterial factor(s) in alveolar lining material of rats. The Journal of Clinical Investigation, 71, 129-141.

Crampton, C.F., Moore, S. & Stein, W.H. (1955) Chromatographic fractionation of calf thymus histone. Journal of Biological Chemistry,215, 787-801.

Crampton, C.F., Stein, W.H. & Moore, S. (1957) Comparative studies on chromatographically purified histones. Journal of Biological Chemistry, 225, 363-368.

Curry, M.C., Hussain, J.I., Smith, C.J. & Allen, J.C. (1980) Identification of a macromolecular inhibitor of in vitro lymophocyte proliferation in human seminal plasma as bound spermine. European Journal of Cell Biology, 21, 180-182.

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. & Jones, K.M. (1969) Data for Biochemical Research, Second Edition, Clarendon Press, Oxford.

Delincee, H. & Radola, B.J. (1978) Determination of isoelectric points in thin-layer isoelectric focusing. Importance of attaining steady-state and role of CO_2 interference. Analytical Biochemistry, 90, 609-623.

Donaldson, D.M. & Marcus, S. (1958) Studies on serum bactericidal activity. Interrelationships of heparin, citrate, protamine and x-irradiation on serum and plasma bactericidal activity against Bacillus subtilis. Journal of Immunology, 81, 292-296.

Donaldson, D.M. & Tew, J.G. (1977) Beta-lysin of platelet origin. Bacteriological Reviews, 41, 501-513.

Dubos, R.J. & Hirsch, J.G. (1954) The antimycobacterial activity of a peptide preparation derived from calf thymus. Journal of Expermental Medicine, 99, 53-63.

Ellestad, G.A., Cosulich, D.B., Broschard, R.W., Martin, J.H., Kunstman, M.P., Marton, G.O., Lancaster, J.E., Fulmor, W. & Lovell, F.M. (1978) Glycocinnamoylspermidine, a new class of antibiotics. 3. The structure of LL-BM123β γ_1, and γ_2. Journal of the American Chemical Society, 100, 2515-2524.

Elsbach, P. (1980) Degradation of microorganisms by phagocytic cells. Review of Infectious Diseases, 2, 106-128.

Elsbach, P. & Weiss, J. (1981) O_2- independent bactericidal systems of polymorphonuclear leukocytes. Advances in Inflammation Research, 2, 95-113.

Elsbach, P. & Weiss, J. (1983) A reevaluation of the roles of the O_2-dependent and O_2-independent microbicidal systems of phagocytes. Reviews of Infectious Diseases, 5, 843-853.

Elsbach, P., Weiss, J., Franson, R.C., Beckerdite-Quagliata, S., Schneider, A. & Harris, L. (1979) Separation and purification of a potent bactericidal/permeability increasing protein and a closely associated phospholipase A2 from rabbit polymorphonuclear leukocytes. The Journal of Biological Chemistry, 254, 11 000-11 009.

Eschenbruch, M. (1980) Investigation into the Nature of Some Mammalian Cationic Antibacterial Proteins. Ph. D. Thesis, University of Waikato, Hamilton, N.Z.

Fair, W.R., Couch, J. & Wehner, N. (1973) The purification and assay of the prostatic antibacterial factor (PAF). Biochemical Medicine, 8, 329-339.

Fair, W.R., Couch, J. & Wehner, N. (1976) Prostatic antibacterial factor, identity and significance. Urology, 7, 169-177.

Fair, W.R. & Parrish, R.F. (1981) Antibacterial substances in prostatic fluid. In: The Prostatic Cell : Structure and Function. Part A Morphologic, Secretory, and Biochemical Aspects. Eds. Murphy, G.P., Sandberg, A.A., Karr, J.P., Alan R. Liss Inc., New York, 247-264.

Fair, W.R. & Wehner, N. (1971) Antibacterial action of spermine: Effect on urinary tract pathogens. Applied Microbiology, 21, 6-8.

Fair, W.R. & Wehner, N. (1971) Further observations on the antibacterial nature of prostatic fluid. Infection and Immunity, 3, 494-495.

Fujii, A., Takita, T., Maeda, K. & Umezawa, H. (1973) Chemistry of Bleomycin XI. The structures of the terminal amines. The Journal of Antibiotics, 26, 398-399.

Furlan, M. & Jericijo, M. (1967) Protein catabolism in thymus nuclei (I) Hydrolysis of nucleoprotein by proteases present in calf thymus nuclei. Biochemica et Biophysica Acta, 147, 135-144.

Gianazza, E., Chillemi, F., Duranti, M. & Righetti, P.G. (1983) Analytical and preparative isoelectric focusing of peptides in immobilised pH gradients. Journal of Biochemical and Biophysical Methods, 8, 339-351.

Ginsburg, I. & Sela, M.N. (1976) Role of leukocytes and their hydrolases in the persistence, degradation and transport of bacterial constituents in tissues: relation to chronic inflammatory processes in staphylococcal, streptococcal, and mycobacterial infections and in chronic peridontal diseases. CRC Critical Reviews in Microbiology, 4, 249-332.

Goldsmith, M.R., Rattner, E.C., Koehler, M.M.D., Balikov, S.R. & Bock, S.C. (1979) Two-dimensional electrophoresis of small molecular weight proteins. Analytical Biochemistry, 99, 33-40.

Gooch, G.T. & Donaldson, D.M. (1974) Reactions of beta-lysin with membranes of Bacillus magaterium.

Gosnell, W.W., Kroger, M., Katz, F. (1975). Improvement of the agar plate method to determine lysozyme. Journal of Milk Food Technology, 38, 457.

Bacteriology,

Gurevitch, J., Rozansky, R., Weber, D., Brzezinsky, A. & Eckerling, B. (1951) The role of spermine in the inhibition of Staphylococcus aureus by human semen. Journal of Clinical Pathology, 4, 360-365.

Hamana, K. (1981) Gel electrophoresis and gel chromatography of calf thymus histones in the presence of a cationic surfactant. Journal of Biochemistry, 90, 1591-1596.

Hamana, K. & Iwai, K. (1974) Gel chromatography and gel electrophoresis of histones in denaturing solvents. Limited dependence on molecular weight. Journal of Biochemistry, 76, 503-512.

Hannappel, E., Davoust, S. & Horecker, B.L. (1982) Isolation of peptides from calf thymus. Biochemical and Biophysical Research Communications, 104, 266-271.

Harold, F.M. (1964) Stabilisation of Streptococcus faecalis protoplasts by spermine. Journal of Bacteriology, 88, 1416-1420.

Hayashi, K., Kugimiya, M., Imoto, T., Fanatsu, M. & Bigelow, G.C. (1968) The inhibitory interaction of cationic detergents with the active center of lysozyme. I. Site of Interaction. Biochemistry, 7, 1461-1466.

Hettinger, T.P. & Craig, L.C. (1970) Edeines. IV. Structures of the antibiotic peptide edeines A_1 and B_1. Biochemistry, 5, 1224-1232.

Hettinger, T.P., Kurylo-Borowska, Z. and Craig, L.C. (1968) Biochem. 7, 4153-4160.

Academy of sciences, 171, 1002-1009.

Ginsburg, I. & Sela, M.N. (1976) Role of leukocytes and their hydrolases in the persistence, degradation and transport of bacterial constituents in tissues: relation to chronic inflammatory processes in staphylococcal, streptococcal, and mycobacterial infections and in chronic peridontal diseases. CRC Critical Reviews in Microbiology, 4, 249-332.

Goldsmith, M.R., Rattner, E.C., Koehler, M.M.D., Balikov, S.R. & Bock, S.C. (1979) Two-dimensional electrophoresis of small molecular weight proteins. Analytical Biochemistry, 99, 33-40.

Gooch, G.T. & Donaldson, D.M. (1974) Reactions of beta-lysin with purified cytoplasmic membranes of Bacillus magaterium. Infection and Immunity, 10, 1180-1182.

Grossowicz, N. & Ariel, M. (1963) Mechanisms of protection of cells by spermine against lysozyme-induced lysis. Journal of Bacteriology, 85, 293-300.

Gurevitch,J., Rozansky, R., Weber, D., Brzezinsky, A. & Eckerling, B. (1951) The role of spermine in the inhibition of Staphylococcus aureus by human semen. Journal of Clinical Pathology, 4, 360-365.

Hamana, K. (1981) Gel electrophoresis and gel chromatography of calf thymus histones in the presence of a cationic surfactant. Journal of Biochemistry, 90, 1591-1596.

Hamana, K. & Iwai, K. (1974) Gel chromatography and gel electrophoresis of histones in denaturing solvents. Limited dependence on molecular weight. Journal of Biochemistry, 76, 503-512.

Hannappel, E., Davoust, S. & Horecker, B.L. (1982) Isolation of peptides from calf thymus. Biochemical and Biophysical Research Communications, 104, 266-271.

Harold, F.M. (1964) Stabilisation of Streptococcus faecalis protoplasts by spermine. Journal of Bacteriology, 88, 1416-1420.

Hayashi, K., Kugimiya, M., Imoto, T., Fanatsu, M. & Bigelow, G.C. (1968) The inhibitory interaction of cationic detergents with the active center of lysozyme. I. Site of Interaction. Biochemistry, 7, 1461-1466.

Hettinger, T.P. & Craig, L.C. (1970) Edeines. IV. Structures of the antibiotic peptide edeines A_1 and B_1. Biochemistry, 5, 1224-1232.

Hettinger, T.P., Kurylo-Borowska, Z. & Craig, L.C. (1970) The chemistry of the edeine polyamine antibiotics. Metabolism and Biological Functions of Polyamines, Annals of the New York Academy of sciences, 171, 1002-1009.

Hibbitt, K.G. (1970) The role of cationic proteins in the natural defense of the mammary gland. In: International Conference on Cattle Disease Proceedings. Eds. McFeely, R.A., Morse, G.E. & Williams, E.I., The American Association of Bovine Practitioners, Stillwater, Oklahoma.

Hibbitt, K.G. & Cole, C.B. (1968) The antibacterial activity of teat canal proteins. Biochemistry Journal, 106, 39P.

Hibbitt, K.G., Cole, C.B. & Reiter, B. (1969) Antimicrobial proteins isloated from the teat canal of the cow. Journal of General Microbiology, 56, 365-371.

Hibbitt, K.G., Brownlie, J. & Cole, C.B. (1971) The antimicrobial activity of cationic proteins isolated from the cells in bulk milk samples. Journal of Hygiene, Cambridge, 69, 61-69.

Hibbitt, K.G. & Benians, M. (1971) Some effect in vivo of the teat canal and effect in vitro of cationic proteins on Staphylococci. Journal of General Microbiology, 68, 123-128.

Hirsch, J.G. (1954) Mechanisms involved in the antimycobacterial activity of certain basic peptides. Journal of Experimental Medicine, 99, 79-88.

Hirsch, J.G. (1956a) Phagocytin: A bactericidal substance from polymorphonuclear leucocytes. Journal of Experimental Medicine, 103, 589-611.

Hirsch, J.G. (1956b) Studies of the bactericidal action of phagocytin. Journal of Experimental Medicine, 103, 613-621.

Hirsch, J.G. (1960a) Antimicrobial factors in tissues and phagocytic cells. Bacterilogical Reviews, 24, 133-140.

Hirsch, J.G. (1960b) Comparative bactericidal activities of blood and plasma serum. Journal of Experimental Medicine, 112, 15-19.

Hirsch, J.G. (1972) The phagocytic defence system. In: Microbial pathogenicity in man and animals. Eds. Smith, H. & Pearce,

Hirsch, J.G., and Dubos, R.J. (1954). Chemical studies on a basic peptide preparation derived from calf thymus. J Exptl. Med. 99: 56-78.

Hodinka, R.L. & Modrzakowski, M.C. (1983) Bactericidal activity of granule contents from rat polymorphonuclear leukocytes. Infection and Immunity, 40, 139-146.

Höltta, E., Jänne, J. & Hovi, T. (1979) Suppression of the formation of polyamines and macromolecules by DL-α-difluoromethilornithine and methylglyoxal bis(guanylhydrazone) in phytohaemagglutinin-activated human lymphocytes. Biochemistry Journal, 178, 109-117.

Hibbitt, K.G. (1970) The role of cationic proteins in the natural defense of the mammary gland. In: International Conference on Cattle Disease Proceedings. Eds. McFeely, R.A., Morse, G.E. & Williams, E.I., The American Association of Bovine Practitioners, Stillwater, Oklahoma.

Hibbitt, K.G. & Cole, C.B. (1968) The antibacterial activity of teat canal proteins. Biochemistry Journal, 106, 39P.

Hibbitt, K.G., Cole, C.B. & Reiter, B. (1969) Antimicrobial proteins isloated from the teat canal of the cow. Journal of General Microbiology, 56, 365-371.

Hibbitt, K.G., Brownlie, J. & Cole, C.B. (1971) The antimicrobial activity of cationic proteins isolated from the cells in bulk milk samples. Journal of Hygiene, Cambridge, 69, 61-69.

Hibbitt, K.G. & Benians, M. (1971) Some effect in vivo of the teat canal and effect in vitro of cationic proteins on Staphylococci. Journal of General Microbiology, 68, 123-128.

Hirsch, J.G. (1954) Mechanisms involved in the antimycobacterial activity of certain basic peptides. Journal of Experimental Medicine, 99, 79-88.

Hirsch, J.G. (1956a) Phagocytin: A bactericidal substance from polymorphonuclear leucocytes. Journal of Experimental Medicine, 103, 589-611.

Hirsch, J.G. (1956b) Studies of the bactericidal action of phagocytin. Journal of Experimental Medicine, 103, 613-621.

Hirsch, J.G. (1960a) Antimicrobial factors in tissues and phagocytic cells. Bacterilogical Reviews, 24, 133-140.

Hirsch, J.G. (1960b) Comparative bactericidal activities of blood and plasma serum. Journal of Experimental Medicine, 112, 15-19.

Hirsch, J.G. (1972) The phagocytic defence system. In: Microbial pathogenicity in man and animals. Eds. Smith, H. & Pearce, J.H., Cambridge University Press.

Hocking, W.G. & Golde, D.W. (1979) The pulmonary-alveolar macrophage. New England Journal of Medicine, 301, 580-587, 639-645.

Hodinka, R.L. & Modrzakowski, M.C. (1983) Bactericidal activity of granule contents from rat polymorphonuclear leukocytes. Infection and Immunity, 40, 139-146.

Hölttä, E., Jänne, J. & Hovi, T. (1979) Suppression of the formation of polyamines and macromolecules by DL-α-difluoromethilornithine and methylglyoxal bis(guanylhydrazone) in phytohaemagglutinin-activated human lymphocytes. Biochemistry Journal, 178, 109-117.

Howard, C.J., Brownlie, J., Gourlay, R.N. & Collins, J. (1975) Presence of a dialysiable fraction in normal bovine whey capable of killing several species of bovine mycoplasmas. Journal of Hygiene, Cambridge, 74, 261-270.

Igarashi, K. & Hirose, S. (1978) Mechanisms of stimulation of polypeptide synthesis by spermidine. Advances in Polyamine Research, 1, 243-254.

Inouye, M. & Pardee. A.B. (1970) Requirement of polyamines for bacterial division. Annals of the New York Academy of Sciences, 171, 901-909.

Jänne, J., Pösö, H. & Raina, A. (1978) Polyamines in rapid growth and cancer. Biochimica et Biophysica Acta, 473, 241-293.

Janson, J.C. (1967) Adsorption phenomena on Sephadex. Journal of Chromatography, 28, 12-20.

Jensen, R.S., Tew, J.G. & Donaldson, D.M. (1967) Extracellular beta-lysin and muramidase in body fluids and inflammatory exudates. Society for Experimental Biology and Medicine, 124, 545-549.

Jollès, P. (1964) Recent developments in the study of lysozymes. Angewandte Chemie, International Edition English, 3, 28-36.

Jollès, P. (1969) Lysozyme: A chapter of molecular biology. Angewandte Chemie, International Edition English, 8, 227-239.

Jollès, P., Bernier, I., Berthou, J., Charlemangne, D., Faure, A., Hermann, J., Jollès, J., Périn, J.-P. & Saint-Blancard, J. (1974) From lysozymes to chitinases: structural, kinetic and crystallographic studies. In: Lysozyme. Eds. Osserman, E.F., Canfield, R.E. & Beychok, S., 31-54, Academic Press, New York.

Johnson, F.B. & Donaldson, D.M. (1968) Purification of Staphylocidal beta-lysin from rabbit serum. Journal Bacteriology, 96, 589-595.

Josephson, R.V., Maheswaran, S.K., Morr, C.V., Jenness, R. & Lindorfer, R.K. (1971) Effect of urea on pI's (isoelectric points) of ampholytes and casein in isoelectric focusing. Analytical Biochemistry, 40, 476-482.

Kido, Y., Harada, T., Yoshida, T., Umemoto, J. & Motoki, Y. (1980) B-1008, a new antibiotic of bacterial origin containing a spermidine moiety. Production, isolation, characterization and biological properties. The Journal of Antibiotics, 33, 791-795.

Laemmli, U.K. (1970) Clevage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

La Force, F.M., Kelly, W.J. & Huber, G.L. (1973) Inactivation of staphylococci by alveolar macrophages with preliminary observations on the importance of alveolar lining material. American Review of Respiratory Diseases, 108, 784-790.

Lehrer, R.I., Ferrari, L.G., Patterson-Delafield, J. & Sorrell, T. (1980) Fungicidal activity of rabbit alveolar and peritoneal macrophages against Candida albicans. Infection and Immunity, 28, 1001-1008.

Lehrer, R.I., Selsted, M.E., Szklarek, D. & Fleischmann, J. (1983) Antibacterial activity of microbicidal cationic proteins 1 and 2, natural peptide antibiotics of rabbit lung macrophages. Infection and Immunity, 42, 10-14.

Lehtovaara, P. (1978) Anomalous migration of leg-haemoglobin on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Biochemistry Journal, 169, 251-253.

Liquori, A.M., (1967) Complexes between DNA and polyamines: a molecular model. Journal of Molecular Biology, 24, 113-122.

Low, T.L.K., Thurman, G.B., McAdoo, M., McClure, J., Rossio, J.L., Naylor, P.H. & Goldstein, A.L. (1979) The chemistry and biology of thymosin. I. Isolation, characterization, and biological activities of thymosin alpha 1 and polypeptide beta 1 from calf thymus. The Journal of Biological Chemistry, 254, 981-986.

Mach, M., Ebert, P., Popp, R. & Oglivie, A. (1982) Compartmentalization of polyamines in mammalian cells. Biochemical and Biophysical Research Communications, 104, 1327-1334.

Macmillan, W.G. & Hibbitt, K.G. (1969) The effect of antimicrobial proteins on the fine structure of Staphylococcus aureus. Journal of General Microbiology, 56, 373-377.

Mager, J. (1955) Influence of osmotic pressure on the polyamine requiremint of Neisseria perflava and Pasteurella tularensis for growth in defined media. Nature, 176, 933-934.

Mann, T. (1964) The biochemistry of semen and of the male reproductive tract. Methuen, London

Matheson, A., Donaldson, D.M. (1968). Alterations in the morphology of Bacillus subtilis after exposure to beta-lysin and ultraviolet light. J. Bacteriol. 95: 1892-1902.

Mezzetti, G., Drusiani, F., Montagnani, G., Capone, G. & Barbiroli, B. (1982) Heat-sensitive spermine-binding factor from peripheral human lymphocyte (41343). Proceedings of the Society for Experimental Biology and Medicine, 169, 280-283.

La Force, F.M., Kelly, W.J. & Huber, G.L. (1973) Inactivation of staphylococci by alveolar macrophages with preliminary observations on the importance of alveolar lining material. American Review of Respiratory Diseases, 108, 784-790.

Lehrer, R.I., Ferrari, L.G., Patterson-Delafield, J. & Sorrell, T. (1980) Fungicidal activity of rabbit alveolar and peritoneal macrophages against Candida albicans. Infection and Immunity, 28, 1001-1008.

Lehrer, R.I., Selsted, M.E., Szklarek, D. & Fleischmann, J. (1983) Antibacterial activity of microbicidal cationic proteins 1 and 2, natural peptide antibiotics of rabbit lung macrophages. Infection and Immunity, 42, 10-14.

Lehtovaara, P. (1978) Anomalous migration of leg-haemoglobin on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Biochemistry Journal, 169, 251-253.

Liquori, A.M., (1967) Complexes between DNA and polyamines: a molecular model. Journal of Molecular Biology, 24, 113-122.

Low, T.L.K., Thurman, G.B., McAdoo, M., McClure, J., Rossio, J.L., Naylor, P.H. & Goldstein, A.L. (1979) The chemistry and biology of thymosin. I. Isolation, characterization, and biological activities of thymosin alpha 1 and polypeptide beta 1 from calf thymus. The Journal of Biological Chemistry, 254, 981-986.

Mach, M., Ebert, P., Popp, R. & Oglivie, A. (1982) Compartmentalization of polyamines in mammalian cells. Biochemical and Biophysical Research Communications, 104, 1327-1334.

Macmillan, W.G. & Hibbitt, K.G. (1969) The effect of antimicrobial proteins on the fine structure of Staphylococcus aureus. Journal of General Microbiology, 56, 373-377.

Mager, J. (1955) Influence of osmotic pressure on the polyamine requiremint of Neisseria perflava and Pasteurella tularensis for growth in defined media. Nature, 176, 933-934.

Mann, T. (1964) The biochemistry of semen and of the male reproductive tract. Methuen, London.

Matherson, A. & Donaldson, D.M. (1970) Effect of beta-lysin on isolated cell walls and protoplasts of Bacillus subtilis. Journal of Bacteriology, 101, 314-317.

Mezzetti, G., Drusiani, F., Montagnani, G., Capone, G. & Barbiroli, B. (1982) Heat-sensitive spermine-binding factor from peripheral human lymphocyte (41343). Proceedings of the Society for Experimental Biology and Medicine, 169, 280-283.

Mezzetti, G., Moruzzi, M.S., Capone, G. & Barbiroli, B. (1980) Polyamine binding by a cytoplasmic factor in the duodenal mucosa of new-born chick. Biochemical and Biophysical Research Communications, 97, 222-229.

Miller, T.E. (1968) Killing and lysis of Gram-negative bacteria through the synergistic effect of hydrogen peroxide, ascorbic acid and lysozyme. Journal of Bacteriology, 98, 949-955.

Miller, J., Michel, J., Bercovici, B., Argaman, M. & Sacks, T. (1976) Studies on the antimicrobial activity of amniotic fluid. American Journal of Obstetrics and Gynecology, 125, 212-214.

Mills, J. & Dublin, D.T. (1966) Some effects of spermine on Escherichia coli. Molecular Pharmacology, 2, 311-318.

Modrzakowski, M.C., Cooney, M.H., Martin, L.E. & Spitznagel, J.K. (1979) Bactericidal activity of fractionated granule contents from human polymorphonuclear leukocytes. Infection and Immunity, 23, 587-591.

Modrzakowski, M.C., Goodrum, K.J. & Spitznagel, J.K. (1981) Bactericidal activity of fractionated granule contents from human polymorphonuclear leukocytes: Studies with leukocytes from normal individuals, Infection and Immunity, 33, 643-645.

Modrzakowski, M.C. & Spitznagel, J.K. (1979) Bactericidal activity of fractionated granule contents from human polymorphonuclear leukocytes: Antagonism of granule cationic proteins by lipopolysaccharide. Infection and Immunity, 25, 597-602.

Morris, D.R. (1978) Polyamine function in rapidly proliferating cells. Advances in Polyamine Research, 1, 105-115.

Morris, D.R. (1981) In: Polyamines in Biology and Medicine. Eds. Morris, D.R. & Marton, L.J., Dekker, New York, 223-242.

Muschel, L.H. & Jackson, J.E. (1963) Activity of the antibody-complement system and lysozyme against rough Gram-negative organisms. Proceedings for the Society of Experimental Biology and Medicine, 113, 881-884.

Noelken, M.E., Wisdom Jr., B.J. & Hudson, B.G. (1981) Estimation of the size of collagenous polypeptides by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Analytical Biochemistry, 110, 131-136.

Odeberg, H. & Olsson, I. (1975) Antibacterial activity of cationic proteins from human granulocytes. Journal of Clinical Investigation, 59, 1118-1124.

O'Farrell, P.H. (1975) High resolution 2-dimensional electrophoresis of proteins. Journal of Biological Chemistry, 250, 4007-4021.

Olsson, I. & Venge, P. (1974) Cationic proteins of human granulocytes. II. Seperation of the cationic proteins of the granules of leukemic myeloid cells. Blood 44 235-246

Osserman, E.F., Lawlor, D.P. (1966). Serum and urinary lysozyme (muramidase) in monocytic and myelomonocytic leukaemia. J. Exp. Med. 124: 921-952.

Panyim, S. & Chalkley, R. (1971) Molecular weights of vertebrate histone exploiting a modified sodium dodecyl sulfate electrophoretic method. Journal of Biological Chemistry, 246, 7557-7560.

Patterson-Delafield, J., Martinez, R.J. & Lehrer, R.I. (1980) Microbicidal cationic proteins in rabbit alveolar macrophages: A potential host defense mechanism. Infection and Immunity, 30, 180-182.

Patterson-Delafield, J., Szklarek, D., Martinez, R.J. & Lehrer, R.I. (1981) Microbicidal cationic proteins of rabbit alveolar macrophages: amino acid composition and functional attributes. Infection and Immunity, 31, 723-731.

Peterson, R.F. (1972) Applicability of acrylamide gel electrophoresis to determination of protein purity. In: Methods of Enzymology. Eds. Hirs, C.H.W. & Timasheff,S.N., Vol 25, 178-182,

Pettersson, A. (1924) Über die thermostabilen bakteriolytischen substanzen des normalserums. Zeitschrift fur Immunitätsforschung, 40, 43-56.

Pharmacia Fine Chemicals (1977) Sephadex. Gel Filtration in Theory and Practice. Uppland Grafiska AB.

Razin, S. & Rozansky, R. (1957) The responsibility of spermine for the antibacterial action of human semen. Journal of Laboratory and Clinical Medicine, 49, 877-881.

Razin, S. & Rozansky, R. (1959) Mechanism of the antibacterial action of spermine. Archives of Biochemistry and Biophysics, 81, 36-54.

Reddy, E.S.P. & Bhargava, P.M. (1979) Seminalplasmin - an antimicrobial protein from bovine seminal plasma which acts in E. coli by specific inhibition of rRNA synthesis. Nature, 279, 725-728.

Reddy, E.S.P., Das, M.R., Reddy, E.P. & Bhargava, P.M. (1983) Inhibition of reverse transcriptases by seminalplasmin. Biochemical Journal, 209, 183-188.

Reisfeld, R.A., Lewis, U.J. & Williams, D.E. (1962) Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature, 195, 281-283.

Olsson, I. & Venge, P. (1974) Cationic proteins of human granulocytes. II. Seperation of the cationic proteins of the granules of leukemic myeloid cells. Blood, 44, 235-246.

Panyim, S. & Chalkley, R. (1969) High-resolution acrylamide gel electrophoresis of histones. Archives of Biochemistry and Biophysics, 130, 337-346.

Panyim, S. & Chalkley, R. (1971) Molecular weights of vertebrate histone exploiting a modified sodium dodecyl sulfate electrophoretic method. Journal of Biological Chemistry, 246, 7557-7560.

Patterson-Delafield, J., Martinez, R.J. & Lehrer, R.I. (1980) Microbicidal cationic proteins in rabbit alveolar macrophages: A potential host defense mechanism. Infection and Immunity, 30, 180-182.

Patterson-Delafield, J., Szklarek, D., Martinez, R.J. & Lehrer, R.I. (1981) Microbicidal cationic proteins of rabbit alveolar macrophages: amino acid composition and functional attributes. Infection and Immunity, 31, 723-731.

Peterson, R.F. (1972) Applicability of acrylamide gel electrophoresis to determination of protein purity. In: Methods of Enzymology. Eds. Hirs, C.H.W. & Timasheff,S.N., Vol 25, 178-182,

Pettersson, A. (1924) Über die thermostabilen bakteriolytischen substanzen des normalserums. Zeitschrift fur Immunitätsforschung, 40, 43-56.

Pharmacia Fine Chemicals (1977) Sephadex. Gel Filtration in Theory and Practice. Uppland Grafiska AB.

Razin, S. & Rozansky, R. (1957) The responsibility of spermine for the antibacterial action of human semen. Journal of Laboratory and Clinical Medicine, 49, 877-881.

Razin, S. & Rozansky, R. (1959) Mechanism of the antibacterial action of spermine. Archives of Biochemistry and Biophysics, 81, 36-54.

Reddy, E.S.P. & Bhargava, P.M. (1979) Seminalplasmin - an antimicrobial protein from bovine seminal plasma which acts in E. coli by specific inhibition of rRNA synthesis. Nature, 279, 725-728.

Reddy, E.S.P., Das, M.R., Reddy, E.P. & Bhargava, P.M. (1983) Inhibition of reverse transcriptases by seminalplasmin. Biochemical Journal, 209, 183-188.

Reisfeld, R.A., Lewis, U.J. & Williams, D.E. (1962) Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature, 195, 281-283.

Righetti, P.G. & Chillemi, F. (1978) Isoelectric focusing of peptides. Journal of Chromatography, 157, 243-251.

Righetti, P.G. & Drysdale J.W. (1976) Isoelectric Focusing. In: Laboratory Techniques in Biochemistry and Molecular Biology. 5, part II. Eds. Work, T.S. & Work, E., North Holland, Amsterdam.

Righetti, P.G., Krishnamoorthy, R., Lapoumeroulie, C. & Labie, D. (1979) Titration curves of polypeptide chains by combined isoelectric focusing-electrophoresis in 8 M urea. Journal of Chromatography, 177, 219-225.

Ritchie, R.F., Harter, J.G. and Bayles, T.B. (1966) J. Lab. Clin. Med. 68, 842-850

Results in Cancer Research, [illegible]

Root, R.K. & Cohen, M.S. (1981) The microbicidal mechanisms of human neutrophils and eosinophils. Review of Infectious Diseases, 3, 565-598.

Rosenthal, S.M., Fisher, E.R. & Stohlman, E.F. (1952) Proceedings for the Society of Experimental Biology and Medicine, 80, 432-434.

Rozansky, R., Gurevitch, J., Brzezinsky, A. & Eckerling, B. (1949) Inhibition of the frowth of Staphylococcus aureus by human semen. Journal of Laboratory and Clinical Medicine, 34, 1526-1529.

Saint-Blanchard, J., Chunzel, P., Mathieu, Y., Perrot, J. & Jolles, P. (1970) Influence of pH and ionic stength on the lysis of Micrococcus lysodeikticus cells by six human and four avian lysozymes. Biochima et Biophys Acta? 220, 300-306.

Salton, M.R.J. (1957) The properties of lysozyme and its action on microorganisms. Bacteriological Reviews, 21, 82-99.

Scales, W.E. & Kluger, M.J. (1984) Low molecular weight substance in peritoneal exudate is antibacterial at febrile temperature. Proceedings of the Society for Experimental Biology and Medicine, 175, 233-236.

Scheit, K.H., Reddy, E.S.P. & Bhargava, P.M. (1979) Seminalplasmin is a potent inhibitor of E. coli RNA polymerase in vitro. Nature, 279, 728-732.

Schollum, L.M., Jarvis, B.D.W. & Bacon, D.J. (1977) Antimicrobial activity of bovine seminal plasma. New Zealand Journal of Agricultural Research, 20, 283-290.

Righetti, P.G. & Chillemi, F. (1978) Isoelectric focusing of peptides. Journal of Chromatography, 157, 243-251.

Righetti, P.G. & Drysdale J.W. (1976) Isoelectric Focusing. In: Laboratory Techniques in Biochemistry and Molecular Biology. 5, part II. Eds. Work, T.S. & Work, E., North Holland, Amsterdam.

Righetti, P.G., Krishnamoorthy, R., Lapoumeroulie, C. & Labie, D. (1979) Titration curves of polypeptide chains by combined isoelectric focusing-electrophoresis in 8 M urea. Journal of Chromatohraphy, 177, 219-225.

Roch, A.M., Quash, G.A., Ripoll, J.P. & Saez, S. (1979). Evidence for natural antibodies (IgG) to polyamines in human sera. Recent Results in Cancer Research, 67, 56-62.

Root, R.K. & Cohen, M.S. (1981) The microbicidal mechanisms of human neutrophils and eosinophils. Review of Infectious Diseases, 3, 565-598.

Rosenthal, S.M., Fisher, E.R. & Stohlman, E.F. (1952) Proceedings for the Society of Experimental Biology and Medicine, 80, 432-434.

Rozansky, R., Gurevitch, J., Brzezinsky, A. & Eckerling, B. (1949) Inhibition of the frowth of Staphylococcus aureus by human semen. Journal of Laboratory and Clinical Medicine, 34, 1526-1529.

Saint-Blanchard, J., Chunzel, P., Mathieu, Y., Perrot, J. & Jolles, P. (1970) Influence of pH and ionic stength on the lysis of Micrococcus lysodeikticus cells by six human and four avian lysozymes. Biochima et Biophys Acta? 220, 300-306.

Salton, M.R.J. (1957) The properties of lysozyme and its action on microorganisms. Bacteriological Reviews, 21, 82-99.

Scales, W.E. & Kluger, M.J. (1984) Low molecular weight substance in peritoneal exudate is antibacterial at febrile temperature. Proceedings of the Society for Experimental Biology and Medicine, 175, 233-236.

Scheit, K.H., Reddy, E.S.P. & Bhargava, P.M. (1979) Seminalplasmin is a potent inhibitor of E. coli RNA polymerase in vitro. Nature, 279, 728-732.

Schollum, L.M., Jarvis, B.D.W. & Bacon, D.J. (1977) Antimicrobial activity of bovine seminal plasma. New Zealand Journal of Agricultural Research, 20, 283-290.

Seale, T.W., Chan, W.Y., Shulka, J. & Rennert, O.M. (1978) Polyamine-conjugated peptide isolated from human plasma. Archives of Biochemistry and Biophysics, 198, 164-174.

Selsted, M.E., Lehrer, R.I., Brown, D.M. & DeLange, R.J. (1983) Multipotent peptides of activated macrophages. Clinical Research, 31, 494.

Shackell, P.J. (1980) Isolation and Characterisation of a Bovine Antibacterial Cationic Peptide. M.Sc. Thesis, University of Waikato, N.Z.

Shannon, P., Curson, B., Twomey, A. & Molan, P.C. (1975) Antibacterial activity of seminal plasma and other tissues. Proceedings of the New Zealand Society of Animal Production, 35, 61-64.

Shannon, P., Twomey, A. & Curson, B. (1974) Antibacterial and antisperm effect of seminal plasma. Proceedings of the New Zealand Society of Animal Production, 34, 61-64.

Shapiro, A.L., Vinuela, E. & Maizel, J.V. (1967) Molecular weight estimation of polypeptide chains by electrophoresis in SDS polyacrylamide gels. Biochemical and Biophysical Research Communication 28, 815-820.

Shivaji, S. (1984) Antimicrobial activity of semen. Trends in Biochemical Sciences, 9, 104-107.

Shoji, J., Sakazaki, R., Wakisaka, Y., Koizume, K., Mayama, M., Matsuura, S. & Tatsumoto, K. (1976) Isolation of a new antibiotic, laterosporamine (Studies on antibiotics from the genus Bacillus. XIII). The Journal of Antibiotics, 29, 390-393.

Silver, S., Wendth, L., Bhattacharyya, P. & Beauchamp, R.S. (1970). Effects of polyamines on membrane permeability. Annals of the New York Academy of Sciences, 171, 838-862.

Skarnes, R.C. & Watson, D.W. (1956a) Characterisation of Leukin: An antibacterial factor from leucocytes active against Gram-positive pathogens. Journal of Experimental Medicine, 104, 829-845.

Skarnes, R.C. & Watson, D.W. (1956b) Characterisation of an antibacterial peptide from calf thymus. Proceedings of the Society for Experimental and Biological Medicine, 93, 267-269.

Skarnes, R.C. & Watson, D.W. (1957) Antimicrobial factors of normal tissues and fluids. Bacteriological Reviews, 21, 273-294.

Spitznagel, J.K. (1979) Antibacterial mechanisms of polymorphonuclear leukocytes. Saliva and Dental Caries, Special Supplement of Microbiology Abstracts, 391-411.

Spitznagel, J.K. (1980) Oxygen-independent antimicrobial systems in polymorphonuclear leukocytes. In: The Reticuloendothelial System: A Comprehensive Treatise, Eds. Friedman, H., Escovar, M. & Reichard, S.M., New York Plenum Press, 2, 355-368.

Stamey, T., Fair, W., Timothy, M. & Chung, H. 1968) Antibacterial nature of prostatic fluid. Nature, 218, 444-447.

Stark, G.R., Stein, W.H. & Moore, S. (1960) Reactions of the cyanate, present in aqueous urea, with amino acids and proteins. Journal of Biological Chemistry, 235, 3177-3181.

Stellwagen, R.H., Reid, B.R. & Cole, R.D. (1968) Degradation of histones during the manipulation of isolated nuclei and deoxyribonucleoprotein. Biochimica et Biophysica Acta, 155, 581-591.

Stevens, L. (1967) Studies on the interaction of homologs of spermine with deoxyribonuclic acid and with bacterial protoplasts. Biochemical Journal, 103, 811-815.

Strominger, J.L. & Ghuysen, J.M. (1967) Mechanisms of enzymatic bacteriolysis. Science, 156, 213-221.

Swank, R.T. & Munkries, K.D. (1971) Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Analytical Biochemistry, 39, 462-477.

Tabor, C.W. & Tabor, H. (1966) Transport systems for 1,4-diaminobutane, spermidine and spermine in Escherichia coli. Journal of Biological Chemistry, 241, 3714-3723.

Tabor, C.W. & Tabor, H. (1976) 1,4-Diaminobutane (putrescine), spermidine and spermine. Annual Review of Biochemistry, 45, 285-301.

Tabor, H. & Tabor, C.W. (1964) Spermidine, spermine, and related amines. Pharmacology Reviews, 16, 245-300.

Tabor, H., Tabor, C.W. & Rosenthal, S.M. (1961) The biochemistry of the polyamines spermidine and spermine. Annual Review of Biochemistry, 30, 579-604.

Tew, J.G., Roberts, R.R. & Donaldson, D.M. (1974) Release of beta-lysin from platelets by thrombin and by a factor produced in heparinized blood. Infection and Immunity, 9, 179-186.

Theil, R. & Scheit, K.H. (1983a) Purification and spectral characterization of seminalplasmin, an antimicrobial protein from bull semen. Hoppe-Seylers Zeitschrift fur Physiologische Chemie, 364, 1003-1009.

Theil, R. & Scheit, K.H. (1983b) Amino acid sequence of seminalplasmin, an antimicrobial protein from bull semen. The EMBO Journal, 2, 1159-1163.

Thomas, J.M. & Hodes, M.E. (1978) Two stage methods for alkaline range analytical isoelectric focusing in thin-layer polyacrylamide gels. Application to cationic proteins in serum. Analytical Biochemistry, 90, 596-608.

Thomas, J.M. & Hodes, M.E. (1981) A new discontinuous buffer system for the electrophoresis of cationic proteins at near neutral pH. Analytical Biochemistry, 118, 194-196.

Tsuboi, M. (1964) The melting temperature of nucleic acid in solution. Bulletin of the Chemical Society of Japan, 37, 1514-1522.

Vakil, J.R., Chandan, R.C., Parry, R.M. & Shanani, K.M. (1969) Susceptibility of several microorganisms to milk lysozymes. Journal of Dairy Science, 52, 1192-1197.

Valkonen, K.H. & Piha, R.S. (1980) Isoelectric focusing and isoelectric points of bovine liver histones. Analytical Biochemistry, 104, 499-505.

Waehneldt, T.V. (1975) Sodium dodecylsulphate in protein chemistry. Bio Systems, 6, 176-187.

Walton, E. (1978) The preparation, properties and action on Staphylococcus aureus of purified fractions from the cationic protein of rabbit polymorphonuclear leucocytes. British Journal of Experimental Pathology, 59, 416-431.

Weber, K. & Osborne, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. The Journal of Biological Chemistry, 244, 4406-4412.

Weiss, J., Beckerdite-Quagliata, S. & Elsbach, P. (1980) Resistance of Gram-negative bacteria to purified bactericidal leukocyte proteins. Relation to binding and bacterial lipopolysaccharide structure. Journal of Clinical Investigation, 65, 619-628.

Weiss, J., Elsbach, P., Olsson, I. & Odeberg, H. (1978) Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. The Journal of Biological Chemistry, 253, 2664-2672.

Weiss, J., Franson, R.C., Beckerdite, S., Schmeidler, K. & Elsbach, P. (1975) Partial characterization and purification of a rabbit granulocyte factor that increases permeability of Escherichia coli. The Journal of Clinical Investigation, 55, 33-42.

Weiss, J., Franson, R.C., Schmeidler, K. & Elsbach, P. (1976) Reversible envelope effects of Escherichia coli W by a highly purified rabbit polymorphonuclear leukocyte fraction. Biochimica et Biophysica Acta, 436, 154-169.

Weiss, J., Victor, M. & Elsbach, P. (1983) Role of charge and hydrophobic interactions in the action of the bactericidal/-permeability-increasing protein of neutrophils on Gram-negative bacteria. Journal of Clinical Investigation, 71, 540-549.

Wilson, A.T. & Rosenblum, H. (1952) The antistreptococcal property of milk. Journal of Experimental Medicine, 95, 25-38.

Yao-Jun, G. & Bishop, R. (1982) Extension of the alkaline ind of a pH gradient in thin-layer polyacrylamide electrofucing gels by addition of N,N,N',N',-tetramethylethylenediamine. Journal of Chromatography, 234, 459-462.

Zeya, H.I. & Spitznagel, J.K. (1968) Arginine-rich proteins of polymorphonuclear leukocyte lysosomes. Antimicrobial specificity and biochemical heterogeneity. Journal of Experimental Medicine, 127, 927-941.

Zeya, H.I. & Spitznagel, J.K. (1969) Cationic protein-bearing granules of polymorphonuclear leukocytes: Separation from enzyme-rich granules. Science, 163, 1069-1071.

Zeya, H.I. & Spitnagel, J.K. (1971) Characterization of cationic protein-bearing granules of polymorphonuclear leukocytes. Laboratory Investigations, 24, 229-236.