AN INVESTIGATION OF THE PHARMACOLOGICALLY-ACTIVE ~~FRACTIONS OF~~ PRINCIPLES IN AMNIOTIC FLUID

by

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ABSTRACT

Human amniotic fluid in late pregnancy (36-42 weeks), possesses antibacterial activity against Bacillus subtilis. High molecular weight substances (> 1000 daltons), present in all amniotic fluids from normal confinements, possess antibacterial activity which is related to the concentration of bound zinc. Antibacterial activity of low molecular weight (LMW) is only detectable in a proportion of specimens; this proportion increases on concentration. LMW activity is reduced in meconium-stained fluids. Amniotic fluids with LMW activity have higher concentrations of bound zinc. This activity is enhanced by manganese or cobalt as well as by zinc. LMW activity, extracted from lyophilised amniotic fluid ultrafiltrates, elutes with an apparent molecular size of 500-600 daltons on gel exclusion chromatography, and displays an apolar nature on reverse phase high pressure liquid chromatography (HPLC).

The LMW activity is extractable from conception products. It is greatest in the placenta, where most activity is found in the microsomal and soluble fractions. Thin layer and lectin chromatography, and the action of proteases, suggest the LMW antibacterial is a peptide.

Human amniotic fluid, at term, also contains hydrophilic myometrial stimulants, which are active on the isolated immature guinea-pig uterus. The oxytocic activity is unchanged in patients with diabetes, hypertension and intra-uterine growth retardation, but is elevated in toxaemic patients. The uterine stimulant is of low molecular weight, gel exclusion chromatography suggesting 500-600 daltons. HPLC resolved two regions of oxytocic activity, the greater proportion of activity residing in the more apolar fraction. The active principle can be distinguished, pharmacologically and

chemically, from physiological smooth muscle stimulants. The oxytocic activity is reduced or abolished by proteases but potentiated by cobalt, magnesium, manganese and zinc. This suggests a peptide nature as only polypeptides with cystinyl bonds are so potentiated.

These findings are discussed with relation to normal and abnormal pregnancies.

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CONTENTS

Title page

Abstract

Dedication

Acknowledgments

Contents

List of Tables

List of Figures

Chapter 1 : Introduction

1.1 Antibacterial activity of human amniotic fluid: relationship to known factors.

1.1.1 Immunoglobulins

1.1.2 Leukin

1.1.3 B-Lysin

1.1.4 Lysozyme

1.1.5 Peroxidase

1.1.6 Transferrin

1.1.7 Low molecular weight antibacterial substances

1.2 Factors affecting expression of antibacterial activity of human amniotic fluid.

1.2.1 Methodological and enviromental factors

1.2.2 Spectrum of susceptible bacteria

1.2.3 Relationship to length of gestation

1.2.4 Variation of activity in population subgroups

1.2.5 Antibacterial activity and bacteriology of human amniotic fluid; the relationship to clinical intra-uterine infection

1.3 Antibacterial activity in related tissues and body fluids.

1.3.1 Vaginal secretions

1.3.2 Cervical mucus

1.3.3 Prostatic secretions

1.4 Uterine stimulant activity in amniotic fluid.

1.4.1 Whole amniotic fluid

1.4.2 Lipid-soluble uterine muscle stimulants of amniotic fluid

1.4.3 Polypeptide uterine muscle stimulants of amniotic fluid

1.5 Summary.

Chapter 2 : Clinical, chemical and pharmacological methods.

2.1 Source and collection of clinical material.

2.1.1 Amniotic fluid

2.1.2 Placental tissues

2.2 Determination of antibacterial activity.

2.2.1 Preparation of bacteria

2.2.2 Antibacterial assay techniques

2.3 Isolated organ preparations.

2.3.1 Guinea-pig uterine horn

2.3.2 Rat uterine horn

2.3.3 Guinea-pig tracheal chain

2.3.4 Human uterine strip

2.4 Physio-chemical determinations.

2.4.1 Chlorhexidine

2.4.2 Inorganic phosphorus

2.4.3 Potassium and Sodium

2.4.4 Protein

2.4.5 Zinc

2.5 Separation and purification techniques.

2.5.1 Ultrafiltration

2.5.2 Lyophilisation

2.5.3 Preparation of crude extract

2.5.4 Size exclusion chromatography

2.5.5 High performance liquid chromatography

2.5.6 Ion exchange chromatography

2.6 Characterisation techniques.

2.6.1 Affinity chromatography

2.6.2 Thin layer chromatography

2.6.3 Chelation studies

2.6.4 Enzymology

2.6.5 Electrophoretic analysis

2.7 Peptide synthesis.

2.7.1 Introduction

2.7.2 Peptide synthesis methods

2.8 Statistical analysis of data.

Chapter 3 : Antibacterial activity of human amniotic fluid.

3.1 Antibacterial activity of whole amniotic fluid.

3.1.1 Selection of assay technique

3.1.2 Antibacterial activity of amniotic fluid

3.2 Relation of antibacterial activity of amniotic fluid to epidemiological factors.

3.2.1 Maturity of pregnancy

3.2.2 Parity of patient

3.2.3 Age of patient

3.2.4 Indication for induction of labour

3.2.5 Source of amniotic fluid

3.3 Studies of the effects of obvious contaminants of amniotic fluid.

3.3.1 Vernix caseosa

3.3.2 Sedimentation products

3.3.3 Blood-stained specimens

3.3.4 Meconium-stained specimens

3.3.5 'Hibitane'-stained specimens

3.4 Discussion.

Chapter 4 : Antibacterial activity of amniotic fluid fractions.

4.1 Antibacterial activity of fractionated amniotic fluid.

4.1.1 High molecular weight activity

4.1.2 Low molecular weight antibacterial activity

4.1.3 Relation between high and low molecular weight antibacterial activity

4.2 Antibacterial activity of low molecular weight fractions.

4.2.1 Antibacterial activity of lyophilised amniotic fluid ultrafiltrates

4.2.2 Preparation of a crude extract with low molecular weight antibacterial activity

4.2.3 Determination of molecular weight of the low molecular weight antibacterial component of amniotic fluid

4.2.4 Separation of components of the acid alcohol extract by high performance liquid chromatography

4.2.5 Ion-exchange chromatography

4.2.6 Summary of purification procedure

4.3 Discussion

Chapter 5 : The effects of environmental factors on the expression of antibacterial activity.

5.1 Relation of antibacterial activity to endogenous zinc and phosphorus.

5.2 Dependence of antibacterial activity on divalent cations.

5.2.1 B-lysin antibacterial activity and the effects of chelating agents

5.2.2 Supplementation with divalent cations, of amniotic fluid treated with chelating agents

5.2.3 Low molecular weight antibacterial activity and the effects of chelating agents

5.2.4 Supplementation with divalent cations, of amniotic fluid extracts treated with chelating agents

5.2.5 Supplementation with radioactive zinc, of acid alcohol extracts treated with dithizone

5.3 Discussion.

Chapter 6 : Characterisation of the low molecular weight component of amniotic fluid.

6.1 Enzymology.

6.2 Affinity chromatography.

6.3 Antibacterial spectrum.

6.4 Thin-layer chromatography

6.4.1 Sugar chromatography

6.4.2 Amino-acid chromatography

6.5 Peptide synthesis.

6.6 Discussion.

Chapter 7 : Source of the low molecular weight antibacterial component of amniotic fluid.

7.1 Investigation of low molecular weight fractions of placenta, amnionic and chorionic membranes for antibacterial activity.

7.1.1 Tissue homogenates and sub-cellular fractionation

7.1.2 High performance liquid chromatographic purification

7.1.3 Gel electrophoretic analysis

7.2 Discussion.

Chapter 8 : General pharmacology.

8.1 Pharmacological activity of amniotic fluid fractions.

8.1.1 Lyophilates of amniotic fluid ultrafiltrates

8.1.2 Lyophilates of gel filtration products

8.1.3 Lyophilates of HPLC fractions

8.2 Investigation of the pharmacological identity of the bronchodilator and oxytocic actions of low molecular weight amniotic fluid components.

8.2.1 Bronchodilator activity

8.2.2 Oxytocic activity

8.3 Discussion.

Chapter 9 : Conclusion.

Chapter 10 : References.

List of Tables:

1. Growth inhibitory activity of amniotic fluid, collected at term, against Gram-positive bacteria.

2. Growth inhibitory activity in amniotic fluid, collected at term, from normal pregnancies, against Gram-negative bacteria.

3. Incidence of antibacterial activity to Escherichia coli and Staphylococcus aureus in amniotic fluid collected in early pregnancy (14 - 20 weeks).

4. Incidence of inhibitory activity to Escherichia coli and Staphylococcus aureus in amniotic fluid collected at term (40 ± 5 weeks) during normal pregnancies.

5. Possible tetra-peptide sequences based on the composition Gln_2, Pro_1, Gly_1.

6. Antibacterial activity of amniotic fluid, as assessed by plate diffusion and tube titration.

7. Antibacterial activity of amniotic fluid, assessed in plain and siliconised plates.

8. The effect of varying degrees of contamination with blood on the expression of antibacterial activity by amniotic fluid.

9. Antibacterial activity of amniotic fluid with and without low molecular weight antibacterial activity.

10. Extraction of antibacterial activity from lyophilised ultrafiltrates of amniotic fluid.

11. Extraction of antibacterial activity from lyophilised ultrafiltrates of amniotic fluid.

12. Extraction of antibacterial activity from lyophilised ultrafiltrates of amniotic fluid.

13. Extraction of antibacterial activity from lyophilised ultrafiltrates of amniotic fluid.

14. Acid alcohol extraction of antibacterial activity from 30 samples of lyophilised ultrafiltrates of amniotic fluid.

15. The relation of antibacterial activity recovered following reverse phase HPLC to loaded activity.

16. Comparison of the separational efficiency of reverse phase HPLC fractionation by echelon and gradient elution.

17. Recovery of antibacterial activity following exposure to ion exchange resins.

18. Extraction of low molecular weight antibacterial activity from amniotic fluid.

19. Amniotic fluid activity in 44 patients and associated levels of total, bound and free, zinc and phosphate.

20. The antibacterial activity expressed by the high molecular weight (B-lysin) fraction of amniotic fluid in the absence or presence of free zinc (1.5 umol/l) and phosphate (150 umol/l).

21. Antibacterial and inactive ultrafiltrates of amniotic fluid; relation to concentrations of zinc and inorganic phosphorus in the original amniotic fluid samples.

22. B-lysin antibacterial activity of amniotic fluid from 8 patients after incubation and reconstitution, and following exposure to chelating agents.

23. Antibacterial activity in amniotic fluid from 8 patients after incubation and reconstitution, re-incubated in the absence or presence of exogenous divalent cations.

24. Antibacterial activity of EDTA-treated amniotic fluids following reincubation in the absence or presence of exogenous divalent cations.

25. Low molecular weight antibacterial activity of amniotic fluid extracts from 8 patients, after incubation at 4^{o}C, and following

exposure to the chelating agents ethylenediamine tetra-actetic acid (EDTA), 1, 10-phenanthroline (o-PA), or diphenylthiocarbazone ('Dithizone').

26. Antibacterial activity of dithizone-treated acid alcohol extracts following re-incubation in the absence or presence of divalent cations.

27. Recovery of antibacterial activity following exposure of acid alcohol extracts to enzyme solution.

28. Recovery of antibacterial activity following exposure of acid alcohol extracts to lectin affinity matrices.

29. Antibacterial activity and slopes of bacterial growth inhibition curves for the low molecular weight antibacterial component of amniotic fluid against a limited spectrum of bacteria.

30. Antibacterial activity of synthesised peptides following incubation in the absence or presence of zinc.

31. Distribution of antibacterial activity from placenta and fetal membranes.

32. Distribution of antibacterial activity through subcellular fractions of human placental homogenates.

33. Purification of oxytocic activity from the low molecular weight fraction of amniotic fluid.

List of Figures:

1. Route of synthesis of a dipeptide from protected amino-acid precursors, by the mixed anhydride method.

2. The frequency distribution of antibacterial activities of human amniotic fluids, displayed in arithmetic and geometric forms.

3. The distribution of slopes derived from the bacterial growth inhibition curves of 122 samples of whole amniotic fluid.

4. Antibacterial activity of amniotic fluid collected between 36 and 42 weeks of pregnancy.

5. The distribution of antibacterial activities of amniotic fluids obtained from nulliparae and multiparae.

6. The distribution of antibacterial activity in amniotic fluid with relation to maternal age.

7. Correlation of total and high molecular weight antibacterial activities in the presence and absence of low molecular weight antibacterial activity.

8. The distribution of antibacterial activities in 29 amniotic fluid ultrafiltrates, displayed in both arithmetic and geometric forms.

9. Determination of the molecular weight of the low molecular weight component of amniotic fluid, with antibacterial activity.

10. Molecular weight calibration curve for Sephadex G15.

11. Separation of the components of an acid alcohol extract by reverse phase high performance liquid chromatography.

12. The distribution of antibacterial activity and weight in HPLC fractions, following separation of acid alcohol extracts by the technique elaborated in figure 11.

13. The relationship of antibacterial activity recovered following HPLC, to the activity of the acid alcohol extract loaded.

14. Duration of elution, of the low molecular weight antibacterial

component in acid alcohol extracts, from the HPLC system and its relation to recovery of antibacterial activity.

15. Refractionation under acid conditions of pooled material with antibacterial activity previously separated by HPLC at neutral pH.

16. Separation of the low molecular weight component of an acid alcohol extract of an amniotic fluid ultrafiltrate by gradient elution.

17. Separation of the components of an acid alcohol extract, by anion exchange chromatography.

18. The distribution of concentrations of zinc and phosphorus in 44 samples of whole amniotic fluid (total values) and in the high (bound) and low (free) molecular weight fractions.

19. Total and high molecular weight antibacterial activities (logarithmic scale); relation to bound zinc concentration.

20. High molecular weight (B-lysin) antibacterial activity of 8 amniotic fluids alone and following treatment with ethylenediamine tetra-acetic acid.

21. Low molecular weight antibacterial activity of 8 amniotic fluid extracts alone, and following treatment with dithizone.

22. Fractionation by reverse phase HPLC of dithizone-treated acid alcohol extracts incubated with radio-zinc.

23. Thin layer chromatography of neutral sugars, amino sugars and amino acids.

24. The relation between estimated peptide concentration and antibacterial activity associated with the active fraction following high performance liquid chromatography of acid alcohol extracts of amniotic fluid ultrafiltrates.

25. Fractionation of peptide product 9 by gradient elution reverse phase HPLC.

26. Rechromatography of HPLC fractionated synthetic peptide.

27. Separation of low molecular weight components of extracts of both the combined mitochondrial and microsomal fractions and the cytosol of human placental homogenates by reverse phase HPLC and subsequent distribution of antibacterial activity.

28. One dimensional SDS-polyacrylamide gel analysis of acid alcohol extracts.

29. Recovery of antibacterial activity from gel slices following PAGE analysis of placental tissue extracts.

30. a) Isotonic comparison of the bronchodilator actions of adrenaline and the low molecular weight fraction of amniotic fluid on the isolated guinea-pig tracheal chain.

b) Comparison of the oxytocic actions of oxytocin and the low molecular weight fraction of amniotic fluid, on the isolated guinea-pig uterine horn.

31. The distribution of the bronchodilator and oxytocic activities of the fraction (III) resulting from gel filtration of lyophilised ultrafiltrates of amniotic fluid.

32. The concentration of potassium as assessed in the eluent following high performance liquid chromatography of lyophilised ultrafiltrates.

33. A comparison of the stimulatory responses elicited by the low molecular weight, apolar oxytocic component of amniotic fluid and bradykinin on the isolated guinea-pig uterine horn.

CHAPTER ONE

Introduction

Many pharmacological agents in present day use are based on biologically active substances which have been identified by analysis of body fluids and tissues.

Interest in the analysis of human amniotic fluid for components with antibacterial activity was prompted by considerable circumstantial evidence that human amniotic fluid is a hostile enviroment to bacteria. Intra-uterine death is rarely complicated by bacterial infection of the amniotic fluid space, which was assumed to be sterile while the fetal membranes remained intact. However, several reports (reviewed by Hawkins & Scane 1983) have indicated that in many apparently normal pregnancies amniotic fluid may contain viable micro-organisms.

Unrecognised chorioamnionitis may be a cause of premature labour. It may also be linked with poor nutrition, as maternal weight gain was often suboptimal in malnourished mothers and neonates were small for gestational age. Antibacterial activity, in amniotic fluid, was either absent or markedly deficient in those patients with a poor diet and those with underweight neonates (Tafari et al. 1977, Ford et al. 1982). Antibiotic chemotherapy may improve the prognosis of these patients.

This introduction will show that amniotic fluid exerts an antibacterial action and that it contains various chemical species which are capable of exerting an antibacterial action. However, several studies have suggested that amniotic fluid does not possess inhibitory activity to bacterial growth (Walsh et al. 1965, Tafari et

al. 1977). Therefore, effects of environmental factors on the antibacterial activity of amniotic fluid will be examined as well.

The presence of smooth muscle stimulating subtances in the liquor amnii has been the subject of study by many investigators. Although most interest has focused on the study of prostaglandins, there is also literature on other oxytocic substances contained in amniotic fluid, which will also be discussed.

The protein and peptide components of amniotic fluid are discussed with respect to their antibacterial action.

1.1.1 Immunoglobulins

Of the classes of immunoglobulins present in humans, three (IgA, IgC and IgM) participate in the resistance of body fluids to infection, and may possess intrinsic antibiotic activity. IgA, IgC and IgM (also IgD and IgG) have all been detected in amniotic fluid (Usategui-Gomez et al. 1966, Galask & Snyder 1970, Curl 1971, Gadow et al. 1974).

Cederqvist et al. (1978) examined the concentrations of a range of immunoglobulins in amniotic fluid, collected throughout the duration of pregnancy. All immunoglobulins were detected from the 11^{th} week of pregnancy. IgA_1, IgA_2, IgD, IgG, all showed a similar pattern: there was an increase in concentrations from weeks 11 to 15 and falling concentrations from 25 to 28 weeks onwards. In contrast, the concentration of IgM remained relatively constant from the 11^{th} week to 35 weeks of pregnancy, but increased to term thereafter.

Secretory IgA (sIgA), which consists of two molecules of IgA, a J (for joining) chain, and a secretory "piece", is thought to have a protective role towards mucosal membranes. Although sIgA is absent from fetus and neonates, Cauchi & Lim (1981) have been able to detect

it in amniotic fluid. The concentration of sIgA was 2 ug/ml at 15-22 weeks and rose continuously to 15-20 mg/ml at term.

Only one study to date (Blanco et al. 1983a) has attempted to relate immunoglobulin concentrations with antibacterial activity of amniotic fluid. It may well be difficult to assess fully the in vivo antibacterial effect of these proteins in an in vitro situation. However, these workers investigated the levels of immunoglobulins in the amniotic fluids of 46 patients with intra-amniotic infection, and in 46 controls, matched for gestational age, the time from rupture of membranes to delivery, and the interval from rupture of membranes to collection of fluid. Only 9 of the 46 fluids from the intra-amniotic infection group and one of the 46 control fluids, had IgM determinable by the assay procedure. The difference in incidence was significant ($P < 0.02$). Similarly 37 of the infection group fluids and 25 of the control fluids had detectable IgA, the difference was also significant ($P < 0.02$). The mean Ig concentration in the infection group was significantly higher than that found in control fluids.

In addition, the antibacterial activity to Escherichia coli, was assessed in 32 intra-amniotic infection fluids and 35 of the controls. Eleven of the infection group fluids, and 24 of the controls were considered inhibitory ($P < 0.02$). However, no (statistically significant) difference was found between the mean IgG level of the inhibitory fluids compared with the non-inhibitory fluids. However, the mean IgG level in the amniotic fluid from patients with intra-amniotic infection was significantly higher than that found in controls.

Overall, it would seem that although the concentrations of certain immunoglobulins (IgA, IgG and IgM) in amniotic fluid are raised in patients with intra-amniotic infection, the antibacterial

activity of amniotic fluid is independent of immunoglobulins. However, the effect of immunoglobulins in amniotic fluid, on leucocyte migration, killer cell activity or macrophage response has yet to be determined. Further investigation is required before postulating any role for the immunoglobulins in amniotic fluid.

1.1.2 Leukin

Leukin is a cationic peptide or protamine derivative, produced during leucocytic degradation (Skarnes & Watson 1956). Like B-lysin, it exhibits a preferential bactericidal activity on Gram-positive bacteria. Ford et al.(1981a) have demonstrated that leukin is present in amniotic fluid in very low concentrations similar to maternal plasma, at 38-40 weeks of pregnancy. The leukin in amniotic fluid may be a consequence of passive diffusion across the placenta. The levels of leukin detected were so low as to lead Ford et al. (1981a) to conclude that "the role of leukin in the non-specific host defence mechanisms of the amniotic fluid is minimal".

1.1.3 B-lysin (bactericidin, plakin)

B-lysin is a collective term for a group of low molecular weight cationic proteins with a preferential lethal action on Gram-positive bacteria (Matheson & Donaldson 1970, Gooch & Donaldson 1974). The primary site of action of B-lysin is considered to be the cytoplasmic membrane (Donaldson & Tew 1977), and thus they have no effect on Gram-negative bacteria with an integral cell wall. Highest bacterial killing rates are observed when B-lysin, lysozyme, and the antibody-complement system function in a cooperative manner.

B-lysin is found predominately in platelets but is also present in serum, plasma and aqueous humour. Rabbit serum B-lysin has been characterised as a divalent cation-dependant polypeptide of 1800 molecular weight (Carrol & Martinez 1981a,b). Rabbit lung macrophage

B-lysin has been shown to comprise of 2 polypeptides, each of 3900 molecular weight (Selsted et al. 1983). The polypeptides differ only in the substitution of an arginine residue (MCP-1) for a leucine residue (MCP-2), consistent with a single base pair change between genomic DNA. Larger forms (8000-12000 molecular weight) have been detected in rabbit granulocytes (Zeya & Spitznagel 1968) and in both guinea-pig and human eosinophils (Gleich et al. 1976). It has also been proposed that a large form which exists (as plakin) in rabbit platelet granules (Weksler & Nachman 1971) is a precursor form (Carrol & Martinez 1981a).

The presence of B-lysin in human amniotic fluid was first observed by Gusdon (1962), who detected B-lysin in 7 of 41 patients in the 36th week of gestation or later. Ford et al. (1977) examined 40 amniotic fluids, 20 from early pregnancy (14-16 weeks) and 20 at term (37-44 weeks). The bactericidal activity of B-lysin towards Bacillus subtilis in amniotic fluid, rose from 4 to 8 units/ml and was distinguished from lysozymic activity by both Seitz filtration and anti-B-lysin antibody. Amniotic fluid levels of B-lysin were higher than those found in serum suggesting that a secretory mechanism exists for this component.

Ford et al. (1981b) also examined the antibacterial activity due to B-lysin, in amniotic fluid collected at term from two groups of different socio-economic status. Amniotic fluids from hispanic patients contained 4 antibacterial units, while those from caucasian patients contained 8 antibacterial units against B.subtilis. Bactericidal activity was removed by bentonite or antibody but not by 1,10-phenanthroline zinc-chelation, while exogenous zinc, cobalt, magnesium or manganese had no effect. Similarly, activity against E.coli was removed by bentonite and antibody. However,

bacteriostatic activity was also removed by 1,10-phenanthroline and restored by zinc (1.5 or 3 umol/l) but not by other divalent cations. Zinc levels in hispanic patients were significantly reduced in comparison with those of caucasian patients. Since B-lysin would not be expected to be active against Gram-negative micro-organisms, it seems likely that the cooperative action of B-lysin, lysozyme and the antibody-complement system seen in other body compartments may be operative in the amniotic fluid space.

The antibacterial spectrum of B-lysin from dialysed amniotic fluid, obtained at term, has been examined (Ford et al. 1982). Zinc-dependent bacteriostasis was observed against Gram-negative enteric bacilli (E.coli, Enterobacter aerogenes, Salmonella typhi and Shigella dysenteriae), Gram-negative bacilli (Bacteroides fragilis, Bacteroides melanogenicus) and Gram-positive cocci (Peptococcus prevotii, Peptostreptococcus foetidus, Staphylococcus aureus, Streptococcus pyogenes, groups B and D streptococci). In contrast, zinc-independent bacteriolysis was observed against aerobic and anaerobic Gram-positive bacilli (Clostridium botulinum, Clostridium tetani, Clostridium welchi and Listeria monocytogenes). As previously described, antibacterial activity was neutralised with a specific antiserum to B-lysin.

Amniotic fluid B-lysin thus seems to play a key role in the prevention of infection of the amniotic cavity.

1.1.4 Lysozyme (Muramidase)

Lysozyme is an endo-N-acetyl muramidase acting upon N-acetyl glucosamine (B-1,4) N-acetyl muramic acid linkages of bacterial cell wall glycopeptides. Alone, it is active against Gram-positive bacteria, whilst in the presence of either antibody and complement, or hydrogen peroxide and ascorbic acid, it is also active against

Gram-negative bacteria.

The first report of the antibacterial activity of human amniotic fluid is widely attributed to Cattaneo (1949), who demonstrated that amniotic fluid would inhibit the growth of Micrococcus lysodiekticus. The finding that the activity was thermostable lead Cattaneo to propose that the factor responsible for the antibacterial action was lysozyme. He also reported that the antibacterial action was antagonised by meconium.

Pommerenke & Taylor (1953) also observed that human amniotic fluid, collected at delivery, exerted a bactericidal action on M.lysodiekticus. A weak action was also observed against S. aureus and Neisseria gonorrheae. The agent in amniotic fluid which inhibited M.lysodiekticus was heat-stable up to 70°C for 30 minutes, and was again proposed to be lysozyme.

Subsequently, there have been several reports on the absolute concentrations of lysozyme present in amniotic fluid. Galask & Snyder (1970) demonstrated that a number of factors were present in amniotic fluid, which could protect the fetus from microbial attack. Using a crude assay, the mean concentration of lysozyme in 3 third-trimester samples of amniotic fluid was found to be 2.5 ug/ml. Bergman et al. (1972) found that amniotic fluid possessed antibacterial activity to a range of Gram-positive and Gram-negative organisms. They too, found that antibacterial activity was related to different antibacterial factors, present in varying combinations. Nine of the 24 third-trimester samples possessed lysozymic activity equivalent to between 0.3 and 2.5 ug/ml.

Several groups of workers have found lysozyme in rising titres during gestation, declining after term. Cherry et al. (1973) found a progressive rise in the mean lysozyme concentration in amniotic

fluid, from 5.1 ug/ml at 14 to 19 weeks of gestation to 18.9 ug/ml at 38 to 40 weeks, and declining thereafter. Similarly, the study performed by Larsen et al. (1974a) showed the mean concentration of lysozyme in amniotic fluid, to rise from 4.8 ug/ml at 28 weeks, to 12.1 ug/ml at 40 weeks, and falling therafter. The same trend was apparent in 8 patients on whom at least 2 amniocenteses were performed. Lysozyme concentrations of amniotic fluid collected in early pregnancy rose from 4.4 ug/ml to 10.4 ug/ml at 21-24 weeks of gestation (Adinolfi et al. 1976). The results of a study by Bratlid & Lindback (1978) were similar to those of Cherry et al. (1973). Lysozyme concentration rose from 6.2 ug/ml at 25 weeks of pregnancy to a peak concentration of 18.3 ug/ml at 39 weeks of gestation. No difference was found in the lysozyme content of amniotic fluid between normal and pathologic pregnancies.

Perhaps the most important contribution was a study by Ford et al. (1977) which examined the relationship between the lysozyme content of amniotic fluid and the antibacterial activity to the Gram-positive micro-organism, B. subtilis. Amniotic fluid was collected from 20 patients at 14 to 16 weeks of gestation and from 20 patients at 37 to 40 weeks of gestation. The mean lysozyme concentration rose from 4.0 ug/ml at 14 weeks, to 14.0 ug/ml in late pregnancy, while antibacterial activity rose from 4 to 8 units/ml. However, egg-white lysozyme, in concentrations up to 20 ug/ml, had no antibacterial effect. Bactericidal activity was removed by both filtration and anti-B-lysin antibody, while lysozymic activity was not.

Thadepalli et al. (1982) analysed amniotic fluid obtained at different periods of pregnancy for antimicrobial factors. Lysozyme concentrations and antibacterial activity to the Gram-negative

bacterium, E. coli were examined. Concentrations of lysozyme were low between 14-20 weeks, high between 36-40 weeks, and decreased after the 40^{th} week of gestation. In general, average lysozyme concentrations at any given gestational age, were higher in the group with inhibitory activity than those in the non-inhibitory group. A large degree of overlap was noted among all periods, and no significant difference between groups was observed.

Overall, it may be concluded that lysozyme can be detected in amniotic fluid from early in pregnancy, and that the concentration then rises, reaching maximal values between 39 and 40 weeks of pregnancy. However, no relationship has been demonstrated between lysozyme concentration and the antibacterial activity of amniotic fluid to Gram-positive micro-organisms (Bergman et al. 1972, Schlievert et al. 1976b, Ford et al. 1977, and Thadepalli et al. 1982).

1.1.5 Peroxidase

This enzyme is an hydrogen peroxide oxidoreductase requiring hydrogen peroxide for oxidation, and halogen ions as cofactors. Its antibacterial mode of action is not clear. It may be due to production of toxic aldehydes from deaminated, decarboxylated amino acids, direct halogenation of microbial proteins, or simply production of toxic chloride ions.

The presence of a peroxidase in amniotic fluid was first demonstrated by Larsen et al. (1974a). Peroxidase was present in 32 of 41 samples of amniotic fluid. Lack of peroxidase activity appeared to be related to gestational age. Peroxidase activity was absent before 32 weeks, but increased during gestation until it reached a peak at 40 weeks, but declined thereafter. Some patients lacked peroxidase even near term.

Using a more sensitive method, peroxidase activity was detected in amniotic fluid samples collected at 11 weeks (Armstrong et al. 1976). Activity rose slowly during pregnancy, but increased dramatically after 34 weeks of gestation. Inhibition data indicated the peroxidase was salivary in origin and was not derived from leukocytes.

Feingold et al. (1979) attempted to relate the antibacterial activity of amniotic fluid to S. aureus, with the concentration of peroxidase. Samples of amniotic fluid were grouped with respect to gestation. Nine of 11 fluids collected between 16 and 20 weeks of gestation were inhibitory, as were 4 of 6 fluids collected between weeks 20 to 28, and 10 of 13 collected between 32 and 42 weeks. Conversely, no peroxidase activity was detected in any of the samples. In view of the fact that both of the previous studies were able to detect peroxidase in amniotic fluid, this finding may be due to methodological differences. Different forms of peroxidase have the ability to metabolise disparate organic acids; it is possible that amniotic fluid peroxidase was not able to act on the particular substrate used in this study.

It is difficult to assess the importance of the peroxidase antibacterial system in amniotic fluid, since hydrogen peroxide concentrations are not known. Certain bacteria (e.g. Lactobacillus acidophilus) produce hydrogen peroxide. In the presence of amniotic fluid peroxidase, this may result in self-destruction. Studies by Saifer et al. (1970), Reid et al. (1971) and Kang & Scanlon (1974) have demonstrated the presence in amniotic fluid of free amino acids which may be utilised by peroxidase to produce toxic aldehydes (Zgliczynski et al. 1968, Strauss et al. 1970, Jacobs et al. 1970 and Paul et al. 1970).

1.1.6 Transferrin

Transferrin is a B_1-globulin, a glycoprotein responsible for transport of plasma-iron to red bone marrow for red blood cell formation. The bacteriostatic role of transferrin is conferred by the ability to chelate iron from its enviroment. Prevention of iron uptake by bacteria is a means of defence against bacterial proliferation and infection.

The bacteriostatic action of transferrin has been demonstrated in vivo against C. welchi (Rogers 1967), Pasturella septica and E. coli (Bullen & Rogers 1969) against which transferrin is also bactericidal. The effect may be abolished by injection of iron compounds in concentrations which saturate transferrin (Bullen et al. 1967, 1968). Increased susceptibility to infection was observed in conditions which involve the production of high levels of iron or iron-containing complexes (e.g. Hematin) in the plasma (Bullen et al. 1972).

Transferrin has a high association constant for iron implying low levels of "free iron".Therefore, pathogenic bacteria must be able to liberate iron from iron proteins in order to proliferate. Several species have the ability to synthesise chelating agents with higher affinity for ferric iron than transferrin, for example 2,3-dihydroxybenzoyl serine by E. coli and P. aerogenes, 2,3-dihydroxybenzoylglycine by B. subtilis (Peters & Warren 1968). Enterochelin is synthesised by Salmonella typhimurium (Pollack & Neilands 1970), E. coli and Klebsiella aerogenes (O'Brien & Gibson 1970), as are the mycobactins by pathogenic Mycobacteria (Snow 1965).

The presence of transferrin in amniotic fluid was first demonstrated by Derrington & Soothill (1961). Their results, expressed as a percentage of the standard serum of the healthy adult

male(!), indicated that the major amniotic fluid proteins were albumin and transferrin, that IgG and ceruloplasmin were present in small quantities and that B-lipoprotein and IgM were absent. α_2-glycoprotein was occasionally present, in minute quantities. The conclusions of their study was that amniotic fluid proteins were derived from the maternal circulation, but that size was not the only determinant for passage across the fetal membranes.

Similarly, analysis of term amniotic fluid proteins by gel electrophoresis (Usategui-Gomez et al. 1966) indicated that albumin accounted for 63% of the total amniotic fluid protein with significant amounts of transferrin (17%) and IgG (7%). Ceruloplasmin and IgA were always present, while IgM, B-lipoprotein and α_2-macroglobulin were absent.

These results were confirmed by Fischbacher & Quinlivan (1970). Galask & Snyder (1970) detected transferrin by immunoelectrophoresis, and showed that, in two samples, transferrin was 11 and 21% saturated with iron. These workers went on to show (Larsen et al. 1973) that the concentration of transferrin remained fairly constant between 25 and 34 weeks, but fell thereafter to term.

Thadepalli et al. (1982) claimed that iron and transferrin levels in amniotic fluid were the most highly reliable predictors of antimicrobial activity. Specifically, the predictive value used was that proportion of transferrin which was present in the form not bound to iron, an inhibitory fluid being indicated by an iron concentration of less than 1.2 ug/ml and by a "free transferrin" concentration of greater than 40%. However, the proportion of transferrin in the "free" form is a dependent variable, which will vary as a function of total transferrin and iron concentrations. These are, in turn, variables which are inversely related to length

of gestation, while antibacterial activity, it is generally agreed, increases with progress of pregnancy. No correlation between antibacterial activity of amniotic fluid and iron/transferrin levels, corrected for gestation, has been shown.

1.1.7 Low molecular weight antibacterial substances

The presence of a low molecular weight factor in amniotic fluid which inhibited the growth of E. coli was demonstrated by Schlievert et al. (1976a). This factor was separated from higher molecular weight substances, such as lysozyme, by passage through an ultrafilter designed to exclude compounds with a molecular weight greater than 10,000. However, amniotic fluid components with lower molecular weights, such as B-lysin, would be expected to co-filter. The antibacterial component could be extracted from the ultrafiltrate with bentonite, a crude cation exchange resin. They showed (Schlievert et al. 1976b) that the antibacterial action of this factor was enhanced by boiling (100°C/15 minutes) partially antagonised by the presence of phosphate in a growth medium (Larsen et al. 1974b), and was dependent on zinc. A concentration ratio of zinc-to-phosphate of more than 1 to 200, was required in the medium for activity (Schlievert et al. 1976a). However, amniotic fluids from early in pregnancy (16-19 weeks) had high concentrations of phosphate and were non-inhibitory, whereas later samples were inhibitory and had lower concentrations of phosphate. This was claimed to support phosphate-sensitivity of the low molecular weight antibacterial component (section 1.2.1), and lead to the proposal that this component was the major antibacterial factor in amniotic fluid. The antibacterial activity of amniotic fluid seems to increase with the duration of pregnancy, while the concentration of inorganic phosphorus seems to decrease (see also Berardi et al. 1979). The equivalent molar

ratios of phoshate-to-zinc <67, 67-135, >135, do not suggest a stochiometric relationship with antibacterial activity. Zinc alone did not exert an antibacterial action, but addition of zinc, in concentration greater than 0.08 ug/ml (1.2 umol/l) rendered a non-inhibitory fluid, inhibitory: the initial zinc concentration was not stated.

The moiety with antibacterial activity was smaller than 10,000 daltons, resistant to enzymatic-degradation by trypsin or pronase (a microbial, neutral protinase) but was inactivated by carboxypeptidase (Schlievert et al. 1976c). The molecular weight of the antibacterial component was estimated by gel filtration to be approximately 700, by comparison with bromophenol blue. Amniotic fluid components, with molecular weights between 2,000 and 10,000 daltons, present in the UM10 ultrafiltrate, were removed by this purification step. Digestion with carboxypeptidase A, and amino acid analysis suggested a hexapeptide but this has not been confirmed by structural analysis or by synthesis. If based on 3 glutamine-glutamate residues, two of glycine and one of lysine, the organic component would have a molecular weight of 630. It was thought that this hexapeptide might form a cationic complex with zinc to form an entity with antibacterial activity (Schlievert et al. 1976b, 1977). Despite the absence of aromatic amino acids (phenylalanine, tyrosine), the fraction from gel filtration had strong optical activity at 280 nm, suggesting co-migratory species.

Sachs & Stern (1979) pooled amniotic fluids collected from 2 groups of patients; group 1 were from second trimester abortions, group 2 at surgical induction of labour at term. Ultrafiltrates of Group 1 pools were inactive against E. coli, S. aureus and haemolytic Streptococci, while those of Group 2 pools were

antibacterial, as determined by turbidometric assessment of overnight cultures. As ion-exchange chromatography of the gel filtration product revealed 2 fractions with strong absorbance at 280 nm, these workers proposed that a "family of peptides" was responsible for the antibacterial action of an amniotic fluid fraction.

The antibacterial activities against E. coli of amniotic fluid from 15 patients with polyhydramnios, was comparable to that of 15 inhibitory amniotic fluids (Larsen et al. 1982). Cell counts were lower than those found in non-inhibitory and nutrient broth controls. It was conceded, finally, that only "a portion of the inhibition observed" in samples of amniotic fluid rather than the entire antibacterial activity of the sample "was due to the low molecular weight inhibitor". A proportion of ultrafiltered amniotic fluids were inhibitory to both B. subtilis and E. coli (Hawkins & Scane 1983). Separation of concentrated amniotic fluid ultrafiltrates on a gel filtration column, produced a fraction with antibacterial activity. In agreement with Schlievert et al. (1976c) and Sachs & Stern (1979), this fraction possessed strong absorbance at 280 nm. The antibacterial component of this fraction was separated from the bacterially inactive compounds with strong optical activity, by reverse-phase high performance liquid chromatography.

Whilst all term amniotic fluids possess antibacterial activity to B. subtilis due to a high molecular weight component(B-lysin), only 1 sample in 5 possessed antibacterial activity associated with a low molecular weight component (Scane & Hawkins 1984). Activities of both species were related to endogenous zinc, particularly in the protein-bound form. Neither high nor low molecular weight forms possessed antibacterial activity which was related to the concentration of inorganic phosphorus.

1.2 Factors affecting the expression of antibacterial activity

1.2.1 Methodological and enviromental factors

Most studies have reported that, on testing amniotic fluid for antibacterial activity, a proportion of fluids were non-inhibitory to the indicator organism. Some studies, however, have reported that all fluids tested were non-inhibitory. Thus amniotic fluids collected in the first trimester of pregnancy were always non-inhibitory. Many fluids collected early in the second trimester (14-20 weeks of pregnancy) were also inactive on testing against Gram-negative indicator organisms, E. coli, B. fragilis (Schlievert et al. 1975b, Thadepalli et al. 1978, Evaldson & Nord 1981).

Walsh et al. (1965) reported that all 5 samples of amniotic fluid collected at term, contained similar numbers of E. coli, streptocci groups B and D, Diptharoids and Candida albicans as broth controls following inoculation and culture. The controls series however exhibited very low growth rates in comparison to other work, suggesting caution when interpreting results. The work of Sarkany & Gaylard (1968) also seemed to indicate lack of antibacterial activity in amniotic fluid. Six samples failed to suppress growth of Lactobacillus acidophilus, Staphylococcus epidermis and Diptheroid bacilli. The fluids were tested in concentrations of 2%, 10% and 50% but not 100%, and the size of the bacterial inoculum was not stated.

The presence of meconium in amniotic fluid is not only associated with fetal trauma but results in a reduction in antibacterial activity. Both Cattaneo (1949) and Florman & Teubner (1969) have demonstrated that the antibacterial activity of amniotic fluid was markedly reduced following addition of meconium; indeed when sufficient meconium was added to amniotic fluid (more than 1% w/v) "it became a very good culture medium". Other studies

(Kitzmiller et al. 1973, Zalewski & Jankowski 1981) have shown that the antibacterial activity is reduced in meconium-stained fluids. Gibbs et al. (1982b) have shown that the incidence of fluids inhibitory to E. coli was 29/39 (74%) while in those fluids stained with meconium, only 8/23 (35%) were inhibitory. The concentrations of zinc and inorganic phosphorus were elevated in meconium-stained amniotic fluids.

One of the major sources of error in microbiological assays can be traced to the media used (de Louvois 1982). The medium used not only affects the growth rate of the indicator organism, it may also affect the activity of the antibacterial being assayed. When the type of growth medium used in an assay is not stated (e.g. Sarkany & Gaylard 1968), comparison of results with other studies may be invalid.

Larsen et al. (1974b) have shown that dilution of amniotic fluid (with saline) to 10% (v/v) permits the growth of a bacterial inoculum, showing that lack of bacterial growth in intact amniotic fluid is not a result of nutritional inadequacy. The addition of a chemically defined medium to a single specimen of amniotic fluid before inoculation resulted in enhanced growth of E. coli when amniotic fluid alone did not support bacterial growth (Larsen et al. 1974c). Addition of the individual components of the defined medium to amniotic fluid, showed that the addition of a phosphate solution could partially reverse the inhibitory capacity of amniotic fluid. This lead to the proposition that phosphate-sensitivity accounted for the inability of previous workers to demonstrate antibacterial activity in amniotic fluid supplemented with growth media containing phosphate. Amniotic fluid which was antibacterial permitted good bacterial growth when supplemented with a nutrient medium (Kitzmiller

et al. 1973). The extent to which the antibacterial activity of amniotic fluid is dependent on zinc concentration and is inhibited by inorganic phophorus, is controversial.

The absolute levels of these amniotic fluid components do not appear to be crucial, although a certain minimum concentration of zinc may be required. Schlievert et al. (1976a) considered that amniotic fluids which were bactericidal had phosphate-to-zinc ratios of less than 100. If the ratio was higher, between 100 and 200, the amniotic fluid was only bacteriostatic. This proposition was supported by Tafari et al. (1977) but countered by the reports of Appelbaum et al. (1979,1980), Berardi et al. (1979), Gibbs et al. (1982b) and Scane & Hawkins (1984).

The study of Berardi et al. (1979) also suggested an alternative explanation for the association of a high phosphate-to-zinc ratio in amniotic fluids with low antibacterial activity and vice versa. Twenty-two samples of amniotic fluid were collected during pregnancy. The samples were tested for antibacterial activity to S. aureus and E. coli, and the concentrations of zinc and phosphorus measured. Linear regression analysis showed that the concentration of phosphorus fell from 23 ug/ml (240 umol/l) at 17 weeks to 12 ug/ml (130 umol/l) at 40 weeks, while the concentration of zinc in amniotic fluid remained constant at 0.11 ug/ml (1.7 umol/l). The incidence of inhibitory activity to either indicator organism did not differ significantly with duration of pregnancy. Thus high concentrations of inorganic phosphorus are found in amniotic fluid collected in early pregnancy when the incidence of antibacterial activity is low, while in late pregnancy the phosphorus concentration is lower and most amniotic fluids have detectible antibacterial activity. These observations are supported by

Schlievert et al. (1977), Thadepalli et al. (1982) and Gibbs et al. (1982b), although Kynast et al. (1978) have suggested that the zinc concentration in amniotic fluid may rise after the 40^{th} week of pregnancy.

In determining the antibacterial activity of amniotic fluid, another variable to be considered is that of the size of the bacterial inoculum. Most studies have added bacteria to amniotic fluid such that the final concentration is of the order of 10^3 to 10^4 viable cells/ml. Galask & Snyder (1968) showed that the inhibitory effect of amniotic fluid could be partially reversed by use of a large inoculum of bacteria. Miller et al. (1976) also found that the inhibitory activity of amniotic fluid was dependent upon the size of the bacterial inoculum. Forty-two of 61 fluids tested were inhibitory with an inoculum of 10^2 colony forming units/ml, while only 15 out of 61 fluids were inhibitory with an inoculum of 10^4 colony forming units/ml.

The *in vitro* test of amniotic fluid for antibacterial activity is at best only a limited indicator of antibacterial activity *in vivo*. As a consequence, some studies (Pommerenke & Taylor 1953, Galask & Snyder 1968, Bergman et al. 1972, Ford et al. 1982 and Buransky et al. 1983) have assessed the antibacterial activity of amniotic fluid *in vitro*, simultanously against a battery of vaginal and non-vaginal pathogens. Even this comprehensive test would not be able to examine the effects of such antibacterial agents as immunoglobulins, leucocyte factors or the tetra-peptide tuftsin which mediate in the humoral response to infection. Leucophilic γ-globulin has been shown to stimulate the activity of neutrophilic granulocytes and macrophages. Only a small fragment of the molecule was responsible for full activity (Najjar 1974). This fragment,

Table 1. Growth inhibitory activity of amniotic fluid, collected at term, against Gram-positive bacteria.

Study	S.aureus	S.haemolyticus	M.lysodiekticus	S.albus	B.subtilis	S.faecalis	S.pyogenes	L.monocytogenes
Pommerenke & Taylor (1953)	1/12	0/9	11/11					
Galask & Snyder (1968)	(+)			(+)	+	(+)	(+)	
Florman & Taubner (1969)	+							+
Bergman et al. (1972)	11/26			16/16		4/24		
Miller et al. (1976)	2/61					0/61		
Appelbaum et al. (1977)	42/85					21/85		
Buransky et al. (1983)	+					+		+
Ford et al. (1982)	+	+			+		+	+
	P.prevotii	P.foetidus	C.botulinum	C.tetani	C.welchi			
	+	+	+	+	+			

Note: Results expressed as a fraction represent the number of inhibitory fluids relative to the total number tested. Those expressed with a + indicate that all fluids tested were inhibitory, while those that are bracketed indicate partial or incomplete inhibition.

Table 2. Growth inhibitory activity in amniotic fluids, collected at term, from normal pregnancies, against Gram-negative bacteria.

Study	*E.coli*	*N.gonorrheae*	*P.aeruginosa*	*P.mirabilis*	*P.morganii*	*E.aerogenes*	*Klebsiella sp.*
Pommerenke & Taylor (1953)	0/11	1/2					
Galask & Snyder (1968)	(+)		(+)	+			+
Bergman *et al.* (1972)	18/27		4/12	12/22	11/13	11/15	10/14
Ford *et al.* (1982)	+					+	+

	B.fragilis	*B.melanogenicus*	*S.dysenteriae*	*S.typhi*
Ford *et al.* (1982)	+	+	+	+

Note: For key see Table 1.

tuftsin, is a cytoactive tetra-peptide (Thr-Lys-Pro-Arg) that is highly specific in its action on the blood polymorphonuclear granulocyte and macrophages of the reticuloendothelial system.

1.2.2 Spectrum of susceptible bacteria

In studies of the antibacterial activity expressed by human amniotic fluid, most studies have confined their investigations to the use of one indicator organism for assessment of growth inhibitory activity. In those studies where this is the case, the majority of studies have favoured the use of E. coli as the indicator organism, while S. aureus and B. subtilis have also been used.

Some studies have investigated the growth inhibitory activity of amniotic fluid, collected at delivery, against a spectrum of bacteria. The results of such studies are summarised for Gram-positive (Table 1) and Gram-negative (Table 2) bacteria. It can be seen that not all bacteria were susceptible to the antibacterials present in amniotic fluid. Also, some amniotic fluids were inhibitory to a particular bacterial species while others were not. This may imply that the concentration of inhibitory factors in amniotic fluid is variable.

The antibacterial activity of 40 samples of amniotic fluid from "patients with imminent fetal dysphasia" was less inhibitory to E. coli, S. aureus, Pseudomonas aeruginosa and Shigella flexneri than 40 samples of amniotic fluid collected from normal deliveries (Zalewski & Jankowski 1981). Meconium-stained fluids demonstrated a "smaller" activity, but the incidence of meconium staining in samples in the groups is not stated. It is therefore dificult to ascertain the relative effect of "imminent fetal dysphasia" or meconium on the antibacterial activity of amniotic fluid.

Table 3. Incidence of antibacterial activity to Escherichia coli and Staphylococcus aureus in amniotic fluid collected in early pregnancy (14 - 20 weeks).

Study		E.coli	S.aureus
Schlievert et al. (19575a)		0/10	-
Evans et al. (1977)		3/13	11/12
Schlievert et al. (1977)		0/20	-
Berardi et al. (1979)		4/9	4/9
Feingold et al. (1979)		-	9/11
Evaldson & Nord (1981)		0/10	-
Thadepalli et al. (1982)		4/20	-
Total		11/82	24/32
	=	13.4%	75.0%

Chi-squared = 38.2 ($P<0.001$)

Table 4. Incidence of inhibitory activity to Escherichia coli and Staphylococcus aureus in amniotic fluid collected at term (40 ± 5 weeks) during normal pregnancies.

Study		E.coli	S.aureus
Pommerenke & Taylor (1953)		0/11	1/12
Bergman et al. (1972)		18/27	11/26
Kitzmiller et al. (1973)		16/17	-
Schlievert et al. (1975a)		25/30	-
Miller et al. (1976)		33/61	2/61
Appelbaum et al. (1977)		21/85	42/85
Evans et al. (1977)		3/10	8/10
Berardi et al. (1979)		3/12	9/12
Feingold et al. (1979)		-	10/13
Guner et al. (1979)		21/29	20/29
Appelbaum et al. (1980)		22/50	57/70
Evaldson & Nord (1981)		10/10	-
Zalewski & Jankowski (1981)		15/40	9/40
Thadepalli et al. (1982)		29/38	-
Mathai et al. (1984)		20/48	24/48
Total		236/468	193/406
	=	50.4%	47.5%

Chi-squared = 0.62 (n.s.)

1.2.3 Relationship to length of gestation

It is possible that antibacterial activity present in amniotic fluid is due to factors which may appear at different stages of pregnancy. No inhibitory activity has been observed in amniotic fluid collected in the first trimester (Thadepalli et al. 1978, Evaldson & Nord 1981). S. aureus, a Gram-positive micro-organism, is sensitive to antibacterial agents (lysozyme, B-lysin) which are inactive against Gram-negative organisms such as E. coli (Appelbaum et al. 1980). Seven studies have reported on the incidence of antibacterial activity to both E. coli and S. aureus in amniotic fluid collected between 14 and 20 weeks of pregnancy. Overall, 13% of samples were inhibitory to E. coli as compared with 75% of fluids that were inhibitory to S. aureus ($P<0.001$, Table 3). This contrasts with the results obtained from 15 studies using samples collected at term. As shown in Table 4, 51% of samples were inhibitory to E. coli while 47% were inhibitory to S. aureus. This difference was not significant.

The study of Berardi et al. (1979) was also of interest, as it demonstrated that the concentration ratio of phosphate-to-zinc declined with advancing pregnancy, from 20 weeks to term ($r = 0.57$, $P < 0.01$). This decline was due solely to the fall in phosphate concentration with pregnancy, as the zinc concentration remained unchanged. There was no difference in the incidence of inhibitory amniotic fluid, to either E. coli or S. aureus.

Overall, Gram-positive organisms (S. aureus, Streptococci, L. monocytogenes, Eubacterium lentum, Peptostreptococcus aerobius were inhibited throughout pregnancy, while Gram-negative organisms (E. coli, B. fragilis) were only inhibited in the third trimester.

1.2.4 Variation of activity in population subgroups

Most studies of the antibacterial activiy of amniotic fluid

have only examined samples obtained from caucasian women. The activity of 53 samples from Ethiopian woman at term was assessed (Tafari et al. 1977). Only 13 samples were inhibitory to E. coli, as compared with 50% of all term fluids reported in Table 4. It must be conceded, however, that in the latter group the incidence varied from 0/11 to 16/17 samples examined. The study by Tafari et al. (1977) also revealed that the concentrations of zinc in the amniotic fluid samples was lower in "underprivileged" women (low income) than "privileged" women. The mean concentrations of zinc were 0.19 ug/ml (2.9 umol/l) and 0.35 ug/ml (5.4 umol/l) respectively.

Amniotic fluid collected from 3 racial groups in Africa, was also assessed for antibacterial activity against S. aureus, E. coli. Streptococcus faecalis, Proteus mirabilis and C.albicans (Appelbaum et al. 1977). Twenty-eight of 37 (76%) fluids from 'white' patients were inhibitory to at least one of the indicator organisms in contrast to 11 of 21 (53%) fluids obtained from Indian patients and only 9 of 27 (33%) fluids from African women.

This last group was investigated further (Appelbaum et al. 1979). The effect of dietary supplementation throughout pregnancy, on the growth supporting property of amniotic fluid at term, was investigated in 100 African women with protein deficient diets. Nine of 24 samples (38%) from control patients (i.e. unsupplemented) possessed inhibitory activity to S. aureus. There was no (significant) change in the inhibition rate for the four treatment groups supplemented with zinc (at either 30 ug/day or 90 ug/day) or protein (animal or vegetable, at 36g/day).

It was proposed that these patients were zinc-deficient on the basis of comparison of concentrations with those of a previous study (Schlievert et al. 1976b), whose results are now regarded as being

elevated over the normal range. Certainly, the concentration of zinc in amniotic fluid could not be considered deficient by caucasian standards. Whilst there was no correlation between activity and phosphate-to-zinc ratio, 17 non-inhibitory fluids became inhibitory following *in vitro* addition of zinc, 1 ug/ml (15 umol/l). Considering both that Abelson & Aldous (1950) found that zinc concentrations greater than 2 ug/ml were inhibitory and that Schlievert *et al.* (1976b) reported that concentrations in excess of 8.5 ug/ml were bactericidal, it is not surprising that Appelbaum *et al.* (1979) found that amniotic fluids which contained zinc at 5 and 10 ug/ml (after additions) were bactericidal.

Similarly, amniotic fluid from 29 Turkish women was assessed for growth inhibitory activity (Guner *et al.* 1979). Inhibition rates were: *E. coli* 21/29, *S. aureus* 20/29 and *C. albicans* 23/29. All 3 micro-organisms were inhibited by 8 fluids, while 18 samples inhibited 2 organisms and 2 samples inhibited one species each. Overall 28 of the 29 samples were inhibitory to one or more organisms.

Following the observation that most fluids from black patients in Ethiopia and South Africa do not inhibit bacterial growth, Appelbaum *et al.* (1980) assessed amniotic fluid antibacterial activity to determine if this was a factor in the differences seen in amniotic fluid infection rates seen in US blacks and US whites. No inter-racial differences were seen in the activities of amniotic fluid from 111 whites and 56 blacks, as assessed against 3 bacterial indicators. Forty-five of 47 fluids collected between 16 and 34 weeks and 101 of 120 fluids collected after 35 weeks inhibited growth of all 3 species: *S. aureus* was most sensitive, being inhibited by 86% of fluids, while *E. coli* was inhibited by 35% of fluids and

Streptococcus agalactiae by 39% of fluids tested. S. aureus was more susceptible to amniotic fluid antibacterials before 35 weeks ($P < 0.05$) while E. coli was more susceptible after 34 weeks ($P < 0.02$).

The antibacterial activity of 32 samples of amniotic fluid from West Virginian women at term, was assessed against 3 strains of E. coli and 1 of S. aureus. Against the original strain, only 13 samples were inhibitory! The other two strains of E. coli appeared to be more susceptible, while S. aureus was intermediate in susceptibility (Larsen et al. 1983).

Mathai et al. (1984) examined the antimicrobial activity of amniotic fluid from 48 South Indian women in the third trimester of pregnancy. All samples were inhibitory to C. albicans and Clostridium perfringens while 46 samples were inhibitory to S. epidermis, 24 to S. aureus and 20 to E. coli, while S. faecalis was not inhibited by any fluid. All women were "well nourished".

Amniotic fluid samples from parous women had greater inhibitory activity against the growth of E. coli than did amniotic fluid from nulliparae ($P < 0.05$). Variation in the antibacterial activity of amniotic fluid, to various microbes, seemed to be less related to the ethnic background of the mother, than to enviromental factors. Thus variation of activity between different population groups was associated with (poor) maternal nutrition and (low) fetal birthweight rather than a specific racial group per se.

1.2.5 Antibacterial activity and bacteriology of human amniotic fluid, and the relationship to clinical intra-uterine infection

A controlled study of intra-uterine infection (IAI) in an indigent population of Mexican-American background has been conducted (Gibbs et al. 1982a). IAI was diagnosed on the basis of fever during

labour ( ) 37.8°C) with simultaneous occurence of two or more of: maternal or fetal tachycardia; uterine tenderness; leukocytosis or foul-smelling liquor. Patients with IAI were matched with controls on the basis of gestational age, interval from rupture of membranes to delivery and interval from rupture of membranes to collection of sample. Patients with IAI had higher mean temperatures and higher mean leukocyte counts than uninfected controls. In 42 of 52 samples from patients with IAI and 16 of 52 controls, more than 100 colony forming units (cfu)/ml were detected in the amniotic fluid ($P < 0.001$). Thirty-six of 52 patients with IAI and 4 of 52 controls, had greater than 100 cfu/ml of a "High-virulence" isolate ($P < 0.001$). These samples were further analysed (Gibbs et al. 1982b) for concentration of inorganic phosphorus and zinc, and for inhibition of growth of E. coli. Samples from 41 patients with IAI had higher mean concentrations of P_i than those of 40 control patients ($P < 0.005$), while zinc levels were comparable. The incidence of meconium-stained amniotic fluid was higher in the infected patients (21/41) than in controls (10/40; $P < 0.02$). However, the mean concentration of P_i in amniotic fluid from patients with IAI was higher than in controls even when meconium-free fluids were compared. Again no difference in mean zinc concentration was observed.

Antibacterial activity to E. coli was assessed in 62 samples; 37 were inhibitory, 25 were non-inhibitory. Only 8 of 23 samples stained with meconium had inhibitory activity as compared with 29 of 39 meconium-free amniotic fluids ($P < 0.01$). No correlation was found between P_i, Zn or P_i/Zn ratio and either intra-amniotic infection or inhibitory activity to E. coli.

The work of Gibbs et al. (1982a,b) was extended by Blanco et al. (1982) to examine the relationship between inhibitory activity

of amniotic fluid and intra-uterine infection. The 50 infected patients were matched for age, parity, gestational age and intervals from rupture of membranes to collection of specimens and to delivery, with uninfected controls. All samples were lyophilised for storage and antibacterial activity tested on reconstitution, alone and following supplementation with zinc or phosphorus. Only 15 of the fluids from infected patients were inhibitory to E. coli, while 34 fluids from control patients were inhibitory. Whilst, in all samples with added phosphate, the viable cell count rose, gradually, to control values in 24 hours, the growth rate was different in each sample of amniotic fluid. Zinc supplementation only affected the growth of E. coli in 3 samples. Amniotic fluids from patients with IAI were less likely to be inhibitory to E. coli than uninfected controls ($P < 0.001$).

The samples were reassessed for inhibition of group B streptococci (Blanco et al. 1983b). By comparison of growth in amniotic fluid in the presence or absence of added phosphate, 8 of 50 infected patients and 18 matched controls were considered inhibitory. Twenty-three samples from each group, however, displayed antibacterial activity when comparing growth in amniotic fluid to that in a broth control. It was concluded that the assay with group B streptococci was "technically more difficult to perform and interpret".

It is unclear whether the inhibitory nature of amniotic fluid prevents intra-amniotic infection, or whether the numbers, virulence or other bacterial properties alter the inhibitory activity of amniotic fluid. The results of the assays are not clear-cut - some samples from infected patients were inhibitory, while some samples from uninfected patients were non-inhibitory.

1.3 Antibacterial activity in related tissues and body fluids

1.3.1 Vaginal secretions

"Vaginal" secretions are a composite of several sources: vaginal secretions; vulval secretions; transudate; cervical mucus and endometrial fluid. The antibacterial activity of vaginal secretions was first reported by Harada (1916) who demonstrated that vaginal secretions of pregnant women were bactericidal, and increased in potency as the pregnancy progressed. Subsequently, Thompson & Khorazo (1935,1940) showed that the vaginal secretions contained lysozyme. Not surprisingly, it was shown that M.lysodiekticus was highly susceptible to the antibacterial effects of vaginal washings (Pommerenke & Taylor 1953). B. subtilis and S. aureus were also slightly inhibited in some cases, while the growth of E. coli, Bacillus cereus, Streptococcus haemolyticus, Bacillus proteus and Streptococcus viridans was not affected.

Several antibacterial proteins (immunoglobulins, lysozyme and transferrin) are present in vaginal secretions, in concentrations comparable to those found in serum (Raffi et al. 1977). The low pH (4.0 - 5.0) of the post menarchal vagina provides a selective advantage to acidophilic bacteria. The maintenance of the low pH is mainly due to the high concentration of lactic acid produced, principally, from vaginal glycogen stores by lactobacilli.

1.3.2 Cervical mucus

Several workers have ascribed antibacterial properties to human cervical mucus. Barton & Wiesner (1945) found that cervical mucus would, in some cases inhibit the growth of S. aureus on agar plates. Pommerenke (1946) showed that cervical mucus inhibited the growth of S. aureus and S. haemolyticus in broth cultures, while the growth of N. gonorrhaeae was enhanced. Using agar diffusion plates,

Pommerenke & Taylor (1953) found completely opposite results : S. aureus, S. haemolyticus and E. coli were not inhibited by cervical mucus which showed weak activity against N. gonorrhaeae and M. lysodiekticus. Lysozyme was proposed as the most probable antibacterial.

Rozanski et al. (1962) examined cervical mucus collected from 110 women between day 10 and day 17 of the menstrual period. Growth of M. lysodiekticus was inhibited by all 40 of the samples tested, as well as by lysozyme. Micrococcus luteus and Bacillus sp. were similarly sensitive to the antibacterial effects of cervical mucus and lysozyme. S. aureus, S. epidermis, S. haemolyticus and E. coli were insensitive to both egg-white lysozyme and cervical mucus.

The antibacterial agent in cervical mucus was heat-labile (while lysozyme was not) and non-dialysable. However cervical mucus exerted the equivalent lytic action against M. lysodiekticus as lysozyme (40 ug/ml) and similarly induced protoplast formation. The conclusion of the study was that cervical mucus contained a substance similar to, but distinct from (egg-white) lysozyme.

It is possible that immunoglobulin and transferrin, present in cervical mucus (Schumacher et al. 1965), may contribute to the antibacterial action.

1.3.3 Prostatic secretions

The prostate is formed, embryologically, as an outgrowth from the proximal end of the urethra and is equivalent to the para-urethral ducts in the female. Urinary tract infections occur 6 to 10 times more frequently in women than in men (Freedman et al. 1965). The reason for this difference may be related to the antibacterial action of prostatic secretions. Lysozyme and spermine have been proposed as being responsible for this activity (Taylor &

Morgan 1952, Gurevitch et al. 1951, Razin & Rozanski 1957,1959), but are not present in concentrations sufficient to exert an antibacterial action (Fair & Wehner 1973). Pronounced antibacterial activity has been observed in the human (Fair et al. 1973, 1976), and is deficient in individuals with chronic prostatitis (Fair et al. 1976).

Early studies indicated that the antibacterial activity of prostatic secretions was confined to a heat-stable, water-soluble fraction containing zinc bound to a low molecular weight peptide (Johnson et al. 1969, Fair et al. 1973, Reed & Stitch 1973). While the concentrations of zinc alone (6.9 mmol/l cf. amniotic fluid 2 -5 umol/l) was sufficient to exert an antibacterial action (Fair & Parrish 1981), the possibility of an antibacterial action due to the peptide was not excluded.

1.4 Uterine stimulant activity in amniotic fluid

1.4.1 Whole amniotic fluid

The stimulant activity of amniotic fluid, collected at delivery, on isolated myometrium was first described by Macciarulo (1931). The activity was not blocked by atropine but was abolished by heating. Hanon & Brunaud (1947a,b) showed that the stimulant effect of amniotic fluid, collected at delivery, was distinct from that produced by acetylcholine, histamine and oxytocin. On lengthy exposure, an "iterative response" was described: the initial contraction was followed by a second, in turn followed by a series of periodic contractions.

The oxytocic action of amniotic fluid was not attributable to calcium, oestrogen, cholesterol, cortisone or progesterone (Hanon et al. 1950,1953). These workers also showed that amniotic fluid from primiparous women was less active than that collected from multiparous women. It was proposed that a uterine-specific substance was present in amniotic fluid, which they termed "Eutocine".

Silquini (1948) affirmed that amniotic fluid collected at term possessed oxytocic activity, which was higher in multiparae than in the primiparous woman. Large doses of amniotic fluid, however, had an inhibitory effect on the spontaneous activity of the isolated guinea-pig uterus. This effect was antagonised by extracts of corpus luteum.

Extensive studies by Stamm (Stamm 1953 a,b,c, 1954), and in collaboration (Stamm & De Watteville 1954) showed that human amniotic fluid would stimulate the isolated uterus of guinea-pig, rabbit and rat. While small doses of amniotic fluid increased spontaneous motility, larger doses produced contracture. He confirmed the results of Hanon & Brunaud (1947a,b) showing a lack of inactivation

of the oxytocic components by boiling at alkaline pH and lack of antagonism of the oxytocic response by doses of atropine or antazoline which abolished responses to acetylcholine and histamine, respectively.

Ogier (1955) showed that amniotic fluid collected in labour contracted the isolated pregnant guinea-pig uterus but not that of the immature guinea-pig. Gabor (1964) demonstrated that amniotic fluid would stimulate, equally, strips of human myometrium from both the pregnant and non-pregnant uterus. Spontaneously-contracting preparations showed an initial contraction followed by increased rhythmic activity, while complete replacement of perfusion fluid with amniotic fluid also caused an iterative response in quiescent uteri or low motility preparations, following a brief latent period.

1.4.2 Lipid-soluble uterine muscle stimulants of amniotic fluid

Hanon et al. (1953) extracted amniotic fluid, obtained at parturition, with acetone, chloroform or ether. The ether extracts were more potent than either of the other two extracts in the stimulation of isolated guinea-pig or rat uterus. The oxytocic activity of the ether extract was differentiated from that of oxytocin.

Precipitation of phospholipids from the amniotic fluid lipids in the ethereal extracts, had no effect on the oxytocic activity. The contractions produced were distinct from those produced by olive oil, oleic acid, sodium oleate or linoleic acid.

The ether-extractable lipids of amniotic fluid collected at surgical rupture of the amniotic membranes for induction of labour were investigated by Ferraris (1958). The amniotic fluid was concentrated five-fold and adsorbed onto Kieselguhr. The dried Kieselguhr was extracted with anhydrous ether, the extracts dried by

evaporation and emulsified at 0.5 to 1% (w/v). The extracts produced mepyramine- and atropine-resistant contractions which gradually assumed a tetanic nature. Boiling the extracts for 30 minutes at pH 8 did not inactivate the oxytocic activity.

Acetone extracts of amniotic fluid obtained at delivery stimulated the isolated uterine cornua of ovarectomised, oestrogen-treated rats (Cuparencu et al. 1962). The reponse was not due to acetylcholine, histamine or 5-hydroxytryptamine, and the substance was resistant to acid hydrolysis. Fatty acid extraction removed the active fraction from solution: Rhodamine B indicating the presence of organic acids.

Hawkins (1962) showed that 100 - 500 ug/ml of an ether extract of amniotic fluid, obtained at surgical rupture of the membranes, was oxytocic to the isolated guinea-pig uterus and to the isolated human uterine strip. The response of the preparation to the extract was not antagonised by mepyramine or atropine.

The extracted material was allowed to partition between 80% aqueous methanol and heptane. Although most of the lipid passed into the heptane phase, the aqueous methanol fraction contained most of the activity. Silicic acid chromatography showed that the methanol extract contained at least four active components. One component appeared to be a free acid as it could be further purified by partitioning into bicarbonate solution.

Amniotic fluid extracts of samples collected between 10 and 20 weeks were comparatively more potent than those collected at term. The extractable lipid content however was less than that obtained from extracts of amniotic fluid collected in late pregnancy. This suggested that dilution of the oxytocic lipids, with inactive lipid, occurs in late pregnancy.

The oxytocic activity of amniotic fluid lipids obtained from spontaneous abortions (8 - 20 weeks) was four times higher than that observed in amniotic fluid obtained from early pregnancy therapeutic terminations (Abrahams & Hawkins 1967). No difference in activity was seen in those samples collected at term and those collected in labour.

In contrast with Hawkins (1962), Abrahams & Hawkins (1967) reported that both oxytocic activity and lipid content increased with the duration of pregnancy. No relation was detected between the amount of oxytocic substances and the occurence of toxaemia of pregnancy. Specimens of meconium-stained amniotic fluid contained greater amounts of lipid-soluble uterine-muscle stimulants than meconium-free fluids.

Bromination or acetylation resulted in complete loss of biological activity (Hawkins & Abrahams 1970), while methylation had little effect on the activity of the oxytocic lipids. Gas liquid chromatography suggested the presence of several long chain unsaturated hydroxy acids. These were also detected in amniotic and chorionic membranes but the highest concentrations of activity were found in the placenta.

The presence of prostaglandins in amniotic fluid was first reported by Karim (1966) who proposed that prostaglandins were intimately involved with parturition. Karim & Devlin (1967) found variable quantities of PGE_1 in all samples of amniotic fluid. Prostaglandins E_2, F_1 and $F_{2\alpha}$ were not detectable in samples collected from quiescent uteri, while concentrations of all four prostaglandins were much higher in those samples obtained from spontaneous abortions and spontaneous labour at term.

Pace-Asciak et al. (1972), Salmon & Amy (1973), Hillier et al.

(1974), Kierse et al. (1974), Johnson et al. (1975), Dray & Frydman (1976) and Tamby Raja et al. (1976), using radio-immuno assays, all detected prostaglandin $F_{2\alpha}$ in amniotic fluid. Although the levels of $PGF_{2\alpha}$ detected in the individual studies show striking differences in magnitude, the consensus of opinion was that the concentration of $PGF_{2\alpha}$ in amniotic fluid rises at the end of pregnancy and continues to rise in an accelerated manner during labour.

Kierse & Turnbull (1973) could not detect PGE_2 in amniotic fluid prior to onset of labour. PGE_2 was detectable in the first stage of labour, the concentration in amniotic fluid rising to delivery. Singh & Zuspan (1974), Tamby Raja et al. (1976) and Dray & Frydman (1976) found similar patterns of results.

The prostaglandin products, found in amniotic fluid, derived from arachidonic acid (PGE_2 and $PGF_{2\alpha}$) predominate over those derived from dihomolinoleic acid (PGE_1 and PGF_1). It is thus consistent that arachidonic acid is present in far greater quantities in the intra-uterine enviroment than dihomolinoleic acid.

1.4.3 Polypeptide uterine muscle stimulants of amniotic fluid

In 1936, Fomina demonstrated that amniotic fluid possessed oxytocic properties comparable to a post-pituitary extract. Specimens collected in early pregnancy were less active than those collected at term.

Reuse & Van den Dreissche (1959) and Van den Driessche (1960) reported that term amniotic fluid would stimulate both isolated guinea-pig uterus and the isolated rat uterus. This action was not attributable to potassium ions, 5-hydroxytryptamine, oxytocin, acetylcholine or histamine, but was very similar to that produced by bradykinin. Delhaye et al. (1967) formally identified that bradykinin was present in amniotic fluid. The concentration of bradykininogen in amniotic fluid decreased with pregnancy. Similarly, kininase activity was more pronounced in early pregnancy than at term (Delhaye et al. 1972).

The oxytocin content of amniotic fluid was determined by radio-immuno assay (Seppala et al. 1972). In late pregnancy and in the absence of contractions, amniotic fluid contained oxytocin at 150 - 800 pg/ml. During labour, the concentration rose to 100 - 1,600 pg/ml ($P < 0.01$). Meconium-stained liquor contained still higher concentrations (1,000 - 14,000 pg/ml).

In 1960, Hunter & Howard reported that both the amniotic fluid and decidua of toxaemic patients in labour, contained a smooth muscle stimulating substance. Absent from the amniotic fluid of normotensive patients, the principle was distinguishable from α-receptor stimulants (adrenaline), 5-hydroxytryptamine, histamine, acetylcholine, potassium ions and oxytocin. The active component was of high molecular weight (> 12,000 daltons), and inactivated by proteolytic enzymes. While it was thermostable between pH 3.3 and pH

8.3, the substance had no antidiuretic action but exerted a strong pressor effect. The active component was thought to be similar to, but different from, angiotonin. Post-partum curettage of pre-eclamptic and eclamptic patients resulted in the return of blood pressure to a normotensive level (Hunter et al. 1961). It was proposed that this return was more rapid than in patients not so treated. These observations were claimed to substantiate their proposal that decidua is a source of the "pressor polypeptide of toxaemia".

Gomel & Hardwick (1966), using the techniques of Hunter & Howard (1960), were unable to reproduce their results. No pressor activity was found in either amniotic fluid or decidual extracts from women with toxaemia of pregnany.

Landesman et al. (1964) collected amniotic fluid and cord blood from both normal and toxaemic patients, and determined the pressor activity of the peptide fraction, in comparison with angiotensin. The average pressor response of amniotic fluid from the 19 patients in the normotensive group was 8.3 mm Hg, with only 2 reponses greater than 10 mm Hg. The average response to amniotic fluid from the toxaemic group was 16.8 mm Hg, with eight responses being greater than 10 mm Hg. With cord blood extracts, the pressor response was 9.3 and 11.5 mm Hg for normotensive and toxaemic patients, respectively.

Seven of the amniotic fluid extracts from the normal group and 1 of the samples from the toxaemic group elicited pressor responses qualitatively different from the angiotensin standard. In addition, recovery after the pressor response exerted by amniotic fluid from the toxaemic patients was slower than that after the standard.

A renin-like enzyme has also been reported in amniotic fluid

(Brown et al. 1964). The concentration in liquor exceeded that found in umbilical vein plasma and peripheral vein plasma. The enzyme was biochemically similar to renin and produced a product pharmacologically similar to angiotensin. However, the plasma renin concentrations in women with severe pre-eclampsia were lower than those of normal pregnant women (Brown et al. 1966).

Goretzlehner & Reithling (1968) published preliminary observations of a study on the presence of a vasoconstrictor substance in toxaemia. Venous blood, urine, amniotic fluid, decidua and placenta were all examined for pressor action. Venous blood consistently produced a pressor reponse, in those samples collected from toxaemic patients while those samples collected from normotensive controls elicited no response. Vasoconstrictor effects were observed in 2 of 4 samples of amniotic fluid from toxaemic patients and not in two samples from normotensive controls.

At least one polypeptide pressor substance was detectable in the dialysate of 7 of 8 toxaemic patients after haemodialysis (Vorne et al. 1974). Four of these patients contained 2 or 3 vasoactive polypeptides. Pressor activity was also observed in the dialysis fluid from a pregnant patient with oedema but not in the post-haemodialysis dialysate of a normal pregnant volunteer. The pressor substance(s) present were distinct from angiotensin II, vasopressin, oxytocin, adrenaline, isoprenaline, 5-hydroxytryptamine and histamine.

1.5 Summary

Although early studies failed to detect antibacterial activity in amniotic fluid, the consensus of contemporary opinion is that such activity does exist and exerts an action on a broad spectrum of

bacteria. The breadth of action may be due to the wide range of antibacterial components found in amniotic fluid. However, it seemed likely that the dominant antibacterial role was played by B-lysin, with the other amniotic fluid components contributing little to this effect. This study sought to examine this aspect and determine the contribution of the low molecular weight antibacterial components to the overall antibacterial effect. Certain enviromental factors have been implicated in the expression of antibacterial activity. The study was designed with this in mind and sought to examine the role of endogenous and exogenous factors associated with the antibacterial action of amniotic fluid.

The nature of low molecular weight antibacterial components of amniotic fluid was the principal focus of attention of this study. Following purification of the component(s), various facets of the chemical nature were to be examined and the component synthesised. The study also sought to determine the site of production of the factor and to examine the spectrum of the antibacterial action. Further, the study was planned to investigate the water-soluble oxytocic action of amniotic fluid and to determine the relationship, if any, with the low molecular weight antibacterial component.

CHAPTER TWO

Clinical, chemical and pharmacological methods

This chapter was designed to show the methodology used in this study. Source and collection methods of clinical material have been delineated. The chapter goes on to show how antibacterial and pharmacological investigations were performed. Chemical and biochemical determinations are elaborated together with the separation, purification and characterisation techniques used. The chapter concludes with sections on peptide synthesis and statistical analysis methods.

2.1 Source and collection of clinical material

2.1.1 Amniotic fluid

Most specimens of amniotic fluid were obtained by rupturing the forewaters with a Drew Smythe's catheter at surgical induction of labour. Some samples were obtained when surgical induction was effected by rupture of the membranes with toothed forceps.

A small number of samples were obtained at occasions other than induction. A few samples were collected in early labour, some were obtained by amniocentesis for assessment of fetal lung maturity, and one sample was obtained by suprapubic drainage, for relief of acute polyhydramnios.

Amniotic fluid was obtained from two sources. The majority of individual samples came from the labour ward at Hammersmith Hospital. Pooled samples together with a few individual samples were obtained from the maternity unit at Northwick Park Hospital.

Contaminants

Amniotic fluid collected at term has, suspended in it, hair, cellular debris and, frequently, vernix caseosa. These contaminants neither possess intrinsic antibacterial activity nor affect the antibacterial activity expressed by amniotic fluid (sections 3.3.1 & 3.3.2). They do, however, block the filters used in preparation of amniotic fluid fractions. Samples of amniotic fluid may also be contaminated with either blood or meconium which interfere with estimations of antibacterial or oxytocic activity. In addition, trace metals are ubiquitous environmental contaminants and care must be taken to exclude them (Reimold & Besch 1978). All surfaces coming into contact with amniotic fluid were acid-washed and all reagents used of 'AnalaR' grade or better.

Routinely, specimens from patients who had received antibiotics in the previous month, and specimens contaminated with blood, meconium or antiseptic cream were excluded. Some samples were retained for comparative purposes. Samples collected by drainage per vaginam are more likely to be contaminated with vaginal flora and secretions, blood, meconium and antiseptic cream than those collected via a Drew Smythe's catheter. No obviously contaminated specimens were used and the concentration of chlorhexidine was determined in those samples which could have been contaminated with antiseptic cream.

Amniotic fluid was thus collected into acid-washed glass containers and refrigerated at 4°C prior to delivery to the laboratory. Samples were then centrifuged at 2000g for 15 minutes at 4°C, and the resultant pale straw coloured supernatant either used immediately for subsequent experimentation or stored at -20°C until use.

2.1.2 Placental tissues

Amnionic and chorionic membranes and placental villous tissue were examined to determine the source of antibacterial activity in amniotic fluid. Tissue types were confirmed by histology. All operations following were performed at 0-4°C unless otherwise stated. On collection, tissues were immersed in phosphate-buffered saline (PBS) pH 7.4, chopped finely, washed until free of blood and shredded in a tissue-press. Placental tissue (but not membranes) required further washing by centrifugation at 300g and resuspension, until free of blood.

The tissues were then blotted dry, weighed, suspended in PBS (9 ml/g) and homogenised in an Ultra Turrax homogeniser (6 x 10 second intervals). Cell debris was removed by centrifugation at 75g. The supernatant was centrifuged at 1000g to yield the nuclear fraction. Mitochondrial and microsomal fractions were prepared by centrifugation at 15000g and 100000g respectively. The resulting particulate fractions were washed, by resuspension and centrifugation, and stored at -20°C. These fractions, lysed in Tris-HCl, 5 mmol/l pH 8.1, together with the cell cytosol were then processed as for amniotic fluid specimens. Antibacterial activity and protein concentrations were assessed as described later.

2.2 Determination of antibacterial activity

All bacteria used were obtained from typed cultures held by the Central Public Health Laboritories, Colindale.

The following bacteria were used:

Aerobes	Bacillus subtilis	NCTC 10400
	Micrococcus luteus	NCTC 8340
	Staphylococcus aureus	NCTC 6571
	Streptococcus faecalis	NCTC 775
	Escherichia coli	NCTC 10418
	Pseudomonus aeruginosa	NCTC 10332
Anaerobes	Clostridium perfringens	NCTC 8237
	Bacteroides fragilis	NCTC 9343

2.2.1 Preparation of bacteria

i) Aerobes

All dried cultures of aerobes were resolved in 0.5 ml Mueller Hinton broth and transferred to a nutrient agar slope. Following overnight incubation at 37°C, the inoculated agar slopes were stored at 4°C. New slopes were inoculated every six months.

a) Bacillus subtilis

Bacillus species are highly sensitive to the majority of antibiotics and have been widely used for their assay (de Louvois 1982). A spore suspension of the micro-organism is used with the advantage that, once prepared, it can be stored at 4°C and used over a period of 6 months (Edberg & Sabath 1980).

The growth from a nutrient slope was suspended in 3 ml sterile saline and transferred to a Roux bottle containing 300 ml Mueller Hinton agar. After incubation for 7 days at 37°C, the resultant growth was washed off with 25 ml sterile distilled water and

incubated at 65°C for 30 minutes to destroy all vegatative cells. The spore suspension was centrifuged, the supernatant discarded and the residual spore suspension washed and resuspended three times with sterile distilled water. The suspension was again heated to 65°C for 30 minutes, centrifuged and resuspended in sterile distilled water. The spore suspension was then distributed into 1 ml aliquots, snap-frozen in dry ice/methanol (-77°C) and stored at -20°C. One sample was thawed, and the number of viable cells determined by the method of Miles & Misra (1935). While in use, the spore suspension was stored at 4°C.

b) Micrococcus luteus, Staphylococcus aureus, Streptococcus faecalis, Escherichia coli and Pseudomonus aeruginosa.

Mueller Hinton broth (500 ml) was inoculated from slope cultures and incubated for 24 hours at 37°C. Following incubation, the bacteria were harvested by centrifugation. The supernatant was discarded and the cells dispersed in sterile glycerol solution (5%). The bacteria were washed twice by centrifugation and resuspension in 50 ml glycerol solution. The cell suspension was divided, snap-frozen and stored at -20°C. As before, a viable cell count was performed.

ii) Anaerobes

a) Clostridium perfringens and Bacteroides fragilis.

The dried cultures were taken up in 0.5 ml thioglycolate broth and incubated at 37°C under an aerobic atmosphere (CO_2:H_2:N_2 10:10:80 %). Clostridium perfringens was grown for 24 hours, while the slower growing B.fragilis was incubated for 48 hours. The cells were harvested by centrifugation and resuspended in sterile glycerol solution (5%). The cells were washed twice by centrifugation and resuspension in glycerol solution, snap-frozen and stored at -20°C. A viable cell count was performed.

2.2.2 Antibacterial assay techniques

Two main types of assay of antibacterial activity were examined; the tube and plate titration methods.

a) Tube titration (after Waterworth 1980)

Two rows of sterile capped tubes were placed in a rack. Four volumes of broth were mixed with the antibacterial powder and one volume added to the first tubes in each row. Doubling dilutions were prepared as follows. Two further volumes of broth were added to the remaining broth, mixed and one volume added to the second tube in each row. This process was repeated until all but the last tubes were filled. One volume of broth was placed in the last tube. Each tube was then inoculated with one drop of an overnight culture of the indicator organism, diluted approximately 1:1000 to give a concentration of approximately 10^6 organisms per ml.

For solutions (ie. amniotic fluid and ultrafiltrates), a slight modification was used. Four rows (A,B,C,D) of tubes were placed in a rack. Four volumes of amniotic fluid were placed in a sterile universal, two volumes were removed into the first tubes of rows A and B. Two volumes of broth were added to the universal, mixed, and two volumes transferred to the second tubes of rows A and B. This procedure was repeated to fill the penultimate tube in each row, to the last tube of which was added 1 volume of broth. The whole procedure was repeated for rows C and D, substituting broth with sterile distilled deionised water. One drop of the diluted indictator organism was again added to each tube.

The solutions were then incubated overnight at 37°C. Growth of bacteria in each tube was assessed either visually or spectrophotometrically. Minimum inhibitory concentrations (MIC's) were expressed as the highest dilution which inhibited growth of the

inoculum. Comparison of MIC's obtained with amniotic fluid diluted with broth and distilled water permitted assessment of suppression of growth inhibitors by broth constituents. This also permitted assessment of the loss of growth inhibitory activity on dilution (Larsen et al. 1974b).

b) Agar plate assay (Garrod et al. 1973)

It has long been accepted that a special agar is required for testing sensitivity of micro-organisms to antibiotics. In the United States, the Food and Drug Administration requires that Mueller Hinton agar is used in the Kirby - Bower sensitivity test. This medium was also recommended for use by the International Collaborative Study. It is also one of the few agars available without added salts, and will support the growth of fastidious micro-organisms.

Agar layers for antibiotic assay must be flat and of a uniform thickness of between 2 and 3 millimetres thick. It is thus essential that the plates used have flat bases; plastic plates (NUNC 1015) have been shown to give results with less variation than using glass plates (Ronsted 1972).

The agar (110 ml) was sterilised by autoclaving (15 psi, 15 minutes), and cooled to 50-55^{o}C. It was then inoculated with the indicator organism, mixed and poured into a plate which had been placed on a levelling table. When set, the plates were dried at room temperature for 30 minutes, and then stored at 0-4^{o}C until use. When using delicate vegatative micro-organisms susceptible to the heat of molten agar (50-55^{o}C), the agar was allowed to set, dried and then flooded with a bacterial suspension.

Before use, plates seeded with B.subtilis (inoculum = 10^{8} viable cells/ml) were exposed to ultraviolet light for 10 minutes to destroy all vegatative cells. Wells were then punched out of the

agar using a cork borer (No 6, outside diameter = 10.5 mm), the agar plugs being removed by vacuum aspiration. Conventional design produces only 36 wells per plate, however 64 wells can be comfortably accomodated on a standard 520 cm^2 plate. The wells were assigned using a random block design (Fisher & Yates 1963) to reduce intra-assay variation. The wells were filled with 100 ul of the test solution and the plate incubated for 1 hour at room temperature to permit pre-diffusion of the antibacterial compound, prior to incubation at 37^{o}C for 16 hours. It is important that a constant incubation period is used, as zones of inhibition may decrease in size with extended incubation times, especially with bacteriostatic drugs.

Zones of inhibition were measured to within 1/4 mm. A good assay system produces circular zones with distinct edges, that can be accurately measured. They should also be as free as possible from partial growth or inner rings of growth. Antibiotic assays are only as good as their dose response curves (Kavanagh 1972). A graphical plot of log concentration against zone size resulted in an apparently linear relationship. Use of a linear regression analysis program on a micro-computer permitted extrapolation of the regression line to intercept the well diameter. This gave a quantitative estimate of the dilution which would be expected to reduce the antibacterial activity of the sample to zero. The reciprocal of this dilution was used to express activity in antibacterial units/ml.

As an example, the following was the result of analysis of an acid alcohol extract for antibacterial activity.

Dilution factor	1/20	1/40	1/80	1/160
Zone of inhibition (mm)	19.0,19.25	17.0,17.0	14.75,14.5	12.5,12.25

Linear regression analysis used the following equation for the dose response curve,

Zone diameter = a + b.log(dilution)

where $a = y - b\bar{x}$

and $b = \frac{(x - \bar{x})(y - \bar{y})}{(x - \bar{x})^2}$

For the case above,

Zone diameter = 28.95 - 7.52.log(DF)

Substitution of the well diameter (10.5 mm) in the above equation, permitted estimation of the dilution (DF_o) which would reduce the antibacterial activity of the extract to zero.

$DF_o = 1/285$

Activity = 285 antibacterial units/ml.

Dose response lines that are too flat lose their accuracy as a large change in drug concentration results in a small change in zone diameter, while those that are too steep do not cover a large enough concentration range to be useful. The presence of unwanted antibacterials may confound the results obtained if the interfering drug(s) is present in a concentration far in excess of that of the antibacterial under test and/or diffuses more rapidly. Regression analysis may detect differences in the slope of the growth inhibition curve and hence reveal the presence of other antibacterial factors.

It has been shown that agar diffusion gives more reliable results than titration in a liquid medium (Reeves & Bywater 1975). In a quality control survey on serum gentamicin assays, use of "doubling dilutions in broth gave the worst results overall". Although both methods were assessed for use (section 3.1.1), only one (the plate diffusion technique) was adopted and used extensively in this study.

2.3 Isolated organ preparations

The animals used were sacrificed by cervical dislocation. Isolated smooth muscle preparations were suspended in a bath of 6 ml volume where contractions and relaxations were monitored using either isometric or isotonic transducers as appropriate. The electrical signals generated by the transducers were passed to a chart recorder.

2.3.1 Guinea-pig uterine horn

On the day prior to the experiment, immature guinea-pigs weighing 150-200 g were exposed to oestrodiol benzoate, 50 ug i.p. (500 ul oestradiol benzoate, 100 ug/ml, in ethanol:normal saline 1:1 Sullivan 1966). The following day, the complete uterine horns were excised and a segment approximately 1.5 cm in length suspended in de Jalon's solution (NaCl 153, KCl 5.6, $CaCl_2$ 0.50, $MgSO_4$ 1.2, $NaHCO_3$ 6.0, dextrose 2.8 mmol/l), after the method of Magnus (1904) which permited exposure of the lumen to the perfusate. The solution was aerated with oxygen containing 5% carbon dioxide, and maintained at 37°C. A dose cycle of 15 minutes was used with a drug contact time of 5 minutes. When using antagonists, the tissue was treated 3 minutes prior to exposure to the agonist.

2.3.2 Rat uterine horn

This tissue was prepared as above, except that the rats (150-200 g) were primed with 20 ug Oestradiol benzoate i.p.

2.3.3 Guinea-pig tracheal chain

These were prepared after the method of Castillo & De Beer (1947) using adult guinea-pigs weighing between 300 and 400 g. After sacrifice, the trachea was exposed from the neck to the point of entry into the lung. The trachea was excised ensuring that no blood was allowed to enter the lumen and placed in Krebs-Henseleit solution (NaCl 118.6, KCl 4.7, $CaCl_2$ 2.6, $MgSO_4$ 1.2, NaH_2PO_4 1.3,

$NaHCO_3$ 24.4, dextrose 11.1 mmol/l; Krebs & Henseleit 1932). Tracheal rings were prepared by slicing transversely through the inter-cartilaginous tissue and assembled into a chain of 6 rings with the muscle strips in alignment. The chains were suspended in Krebs-Henseleit solution maintained at 37°C and aerated with oxygen containing 5% carbon dioxide. A stabilization and recovery period of one hour was allowed prior to testing. A drug contact time of 3 minutes was used with a dose cycle of 15 minutes. This tissue has the advantage that, after stabilization, it possesses intrinsic tonus and thus may be used to demonstrate both contractile and relaxatory effects.

2.3.4 Human uterine strip

Human myometrium was obtained following surgical removal of the uterus. Myometrium was not taken from uteri with dense fibroid formations. Strips of myometrium were prepared by the method of Chambers & Pickles (1958). The strips were suspended in Krebs-Henseleit solution at 37°C and aerated with oxygen containing 5% carbon dioxide. A dose cycle of 15 minutes with a drug contact time of 5 minutes was used.

2.4 Physico-chemical determinations

2.4.1 Chlorhexidine

The method of Rowcroft (1977) was used to determine the concentration of "Hibitane" (chlorhexidine gluconate) in solution, modified both for use with smaller volumes and so as to improve sensitivity and reduce variation.

Reagents: 1. "Hibitane" acetate standard, stock solution 5 mg/ml

2. Cetrimide/isopropyl alcohol solution.
   Cetrimide:isopropyl alcohol:water, 5:2:5
3. Sodium hydroxide solution 1.33 mol/l
4. Alkaline sodium hypobromide solution (1% w/v available bromine). Bromine (0.275 ml) in 75 ml sodium hydroxide solution, 1.33 mol/l

A standard curve was generated by preparing solutions of "Hibitane" acetate in the range 0-100 ug/ml. Duplicate 1.0 ml volumes of the standard solutions were transferred to a water bath maintained at 20°C, together with appropriate volumes of test solutions. Cetrimide/isopropyl alcohol solution (0.30 ml), distilled deionised water (2.6 ml) and alkaline sodium hypobromite solution (0.10 ml) were added and the tubes thoroughly mixed. After incubation for 25 minutes, the optical density of the solution at 480 nm was measured against a reagent blank.

The calibration curve was used to determine the "Hibitane" content of the aliquot and hence the "Hibitane" concentration of the initial solution in terms of "Hibitane" acetate. The value obtained was multiplied by 1.435 to obtain a result in terms of "Hibitane" gluconate.

2.4.2 Inorganic phosphorus

The concentration of inorganic phosphorus in solution was estimated by the molybdenum blue method (Dryer et al. 1957, Laurence 1974), as modified for use with small volumes.

Reagents: 1. Potassium dihydrogen orthophosphate, stock 25 mmol/l

2. Sodium dodecyl sulphate, 1% w/v

3. Acid molybdate reagent. Ammonium molybdate tetrahydrate (2.5 g) in 100 ml sulphuric acid (3.0 mol/l)

4. p-semidine reagent. N-phenyl-p-phenylenediamine hydrochloride (25 mg), wetted with 95% ethanol (0.2 ml), dissolved in 100 ml sodium hydrogen sulphate solution (sodium metabisulphite 5.24 g/l). After stirring and filtering, the reagent was stored for up to 1 month at 4°C.

A standard curve was generated by preparing solutions of potassium dihydrogen phosphate in the range 0-1000 umol/l. Two hundred microlitres of these solutions were transferred in duplicate to test-tubes, together with 200 ul of the test solutions. Sodium dodecyl sulphate (1.2 ml) was then added and each tube thoroughly mixed. Acid molybdate reagent (100 ul) was then added to each tube which was again thoroughly mixed. Finally the p-semidine reagent (1.5 ml) was added and the tubes thoroughly mixed again. After incubation for 1 hour, the optical density of the solutions was measured at 770 nm.

2.4.3 Potassium and Sodium

The concentrations of potassium and sodium in solution were determined by use of ion-selective electrodes, in a Beckman system E2A analyser.

2.4.4 Protein

The protein concentrations in both amniotic fluid and the placental extracts were estimated by the method of Lowry et al. (1951).

Reagents: 1. Bovine serum albumin, stock solution 10 mg/ml

2. Sodium potassium tartrate, 2.0%

3. Copper sulphate, 1.0%

4. Sodium carbonate 2.0%, in 0.1 ml/l sodium hydroxide

5. Folin - Ciocalteau reagent, 40%

Solution A; Reagent 2:Reagent 3:Reagent 4, 1:1:100 v/v

The protein standard was diluted to give a working range of 0-250 ug/ml. To 0.2 ml protein solution (standard or test solution) was added 3.0 ml solution A. After mixing, the solution was allowed to stand for 15 minutes. Folin-Ciocalteau reagent (0.2 ml) was then added, the solution mixed and allowed to stand for a further 30 minutes. The optical density of the solution at 750 nm was measured and the concentration of protein in the test solution determined by comparison with the standard curve.

2.4.5 Zinc

The concentration of zinc in solution was determined by flame aspiration atomic absorption spectroscopy. A Perkin Elmer 290B spectrophotometer together with a multi-element, hollow cathode lamp was used. The following conditions applied:

Lamp current	6 mA
Slit width	0.7 nm
Acetylene pressure	0.6 bar
Acetylene flow	13.5
Air flow	11.7
Damping factor	2
Detection wavelength	214 nm
Aspiration rate	5-6 ml/min

A zinc solution of certified concentration (5mmol/l;Spectrosol BDH) was used to prepare a standard curve in deionised, distilled water (resistivity > 15 Megohm/cm), with a working range of 0-10 µmol/l. The concentration of zinc in the test solutions was determined by comparison with the standard curve. Total zinc levels were measured in whole amniotic fluid, and the free levels in the ultrafiltrates. Protein-bound concentrations were obtained by subtraction.

2.5 Separation and purification techniques

2.5.1 Ultrafiltration

Amniotic fluid, cell cytosol and lysed sub-cellular fractions of placental tissues were all ultrafiltered, and the ultrafiltrate used for subsequent determinations. Ultrafiltration was performed, at 4°C under nitrogen at 3 bar, in an Amicon cell; Model 402 (for volumes greater than 50 ml) and a Model 52 cell for smaller volumes. The filtration membrane used had an exclusion limit of 1000 daltons, which permitted separation of the low molecular weight (ultrafilterable) antibacterial activity from the antibacterial components with higher molecular weights. There was no appreciable loss of either ultrafilterable or non-filterable antibacterial activity, by binding to the ultrafiltration membrane.

Initial experiments used UM2 membranes which were superceded by a more robust YM2 membrane (both made by Amicon). There was no difference in recovery of either ultrafilterable or non-filterable antibacterial activity between these two filter types. After use, the membranes were washed twice with distilled water, followed by 1 mol/l hydrochloric acid, washed twice in deionised water and stored in 10% aqueous methanol.

2.5.2 Lyophilisation

All ultrafiltrates were snap-frozen and lyophilised for storage. Small volumes (less than 10 ml) were shell-frozen in glass universals, while larger volumes (20-500 ml) were shell-frozen in round bottomed flasks. Shell-freezing was effected by rotation of the vessel containing the ultrafiltrate, in methanol which had been chilled by use of solid carbon dioxide (dry ice,"cardice") to temperatures below -30°C.

Lyophilisation was performed by exposure of the frozen

material to vacuum (< 200 mT) using a Chem Lab SB6 freeze drier.

2.5.3 Preparation of crude extract

A variety of extraction techniques have been used to obtain components of interest from physiological solutions. Peptides have generally been recovered by the use of a form of acid-alcohol extraction. Gomes (1955) used n-butanol to extract a bradykinin-like component from urine, which had been acidified with hydrochloric acid and saturated with sodium chloride. The active component was precipitated from the butanol extract by treatment with diethyl ether. Similarly, Morris & Robinson (1964) used ethanol, acidified with hydrochloric acid, to extract angiotensin from human blood. The extract was partially purified by precipitation from methanol with diethyl ether.

Byfield et al. (1976) used butanol, acidified with acetic acid, to extract calcitonin from rat thyroid. The crude extract was partially purified by partitioning between formic acid and diethyl ether. Ethanol/hydrochloric acid extraction was used by Franklin et al.(1976) and Daughaday et al.(1980) to recover somatomedin from serum. Girgis et al. (personal communication, 1985) used a tri-acid (formic/trifluoroacetic/hydrochloric acids) extraction technique in the purification of calcitonin-gene-related peptide.

It was determined that the low molecular weight antibacterial factor could be removed from the lyophilate of amniotic fluid ultrafiltrates, by extraction into acid alcohol using the methods of Byfield et al. (1976). It was decided if possible that the components of the extraction medium should be volatile. Accordingly, amniotic fluid ultrafiltrates were shell-lyophilised and a sample removed for assay of antibacterial activity. The remaining lyophilate was extracted three times with 2 ml of extracting medium

(methanol-acetic acid-water, 90:10:5, v/v; sodium chloride 1%, w/v) per 100 ml of original ultrafiltrate. Insoluble material was removed by centrifugation, the extracts combined, evaporated under nitrogen and dried *in vacuo*.

Further purification, again using the method of Byfield *et al.* (1976) was attempted. The extract was partitioned between 0.1 mol/l formic acid and diethyl ether. The phases were separated, dried *in vacuo* and assayed for antibacterial activity. The effect of simple contact with organic solvent was also investigated. Aliquots of an acid alcohol extract were dried *in vacuo*, and incubated with solvent for 1 hour at 4°C. The solvent was removed by evaporation and the antibacterial activity of the extract reassessed.

2.5.4 Size exclusion chromatography

As a solute passes down a chromatographic bed, its movement depends both on the bulk flow of the mobile phase and upon the Brownian motion of the solute molecules, which causes their diffusion into and out of the stationary phase. The separation in size exclusion chromatography depends on the differing abilities of the various sample molecules to enter pores within the stationary phase. Large molecules which never enter the stationary phase elute very quickly. Smaller molecules move more slowly through the column as they spend a proportion of the elution time within the gel matrix. Molecules are therefore eluted in order of decreasing molecular size.

1. Choice of gel type

The material to be separated had a nominal maximum molecular weight of 1000 daltons (section 2.5.1), which placed a limit on the selection of useful gel types. Sephadex G10 and G15 (Pharmacia) have effective resolution ranges up to 700 and 1500 daltons respectively, while Biogel P2 (Biorad) has an effective range up to 2000 daltons.

Sephadex G10 was used by Sachs & Stern (1979) and Biogel P2 by Schlievert et al.(1976c) for the purification of low molecular weight material from amniotic fluid.

2. Choice of column

a) Dimensions

In general terms, the length of the column is determined by the resolution required and the diameter by the sample capacity. Resolution of separated zones increases as the square root of column length, thus columns should be as long as possible to obtain the best resolution. For analytical purposes, maximal resolution is obtained when the volume of sample is restricted to 1 - 5% of the bed volume (V_t). Thus, if the maximum load = 2 ml ($\equiv$ 5% V_t) then the bed volume = 40 ml.

$$\text{Therefore } V_t = Pi\ r^2\ h = 40 \text{ ml}$$

where r is the internal radius of the column (0.45cm) and h is the length of the gel bed

$$\rightarrow h = 62.9 \text{ cm}$$

Thus minimum bed length, for a maximum sample load of 2 ml, is 63 cm.

b) Flow rate

$$\text{Linear flow rate, } V = k_o . \frac{\Delta p}{h} \text{ ml.cm}^{-2}.\text{hr}^{-1}$$

where k_o is the specific permeability of the gel, which for Sephadex G10 = 19, and for G15 = 18, and Δp is the pressure drop (expressed in cm H_2O).

Thus for a 63 cm column with a constant pressure head of 100 cm, theoretical flow rates for G10 and G15 are 30.1 and 28.6 ml/hr respectively (0.50 and 0.48 ml/min). However, optimal resolution is achieved with a linear flow rate of 2 ml/cm ^{2}/hr, although flow rates up to five times faster may be used without deterioration. Thus

for a 0.9 cm column, maximal resolution with minimal duration is obtained with flow rates between 1.6 and 8.1 ml/hr.

3. Choice of eluent

An ionic strength of at least 0.02 is required to prevent ionic interaction of solutes with the gel matrix. However, both Schlievert et al. (1976c) and Sachs & Stern (1979) used distilled water as eluent. Since non-ideal behavior is minimised with use of near neutral eluents and the eluent was to be lyophilised after chromatography, the volatile buffer ammonium acetate was used, adjusted to pH 7.0 with ammonium hydroxide solution. Homogeneity of eluent flow within the gel matrix was confirmed using Blue Dextran 2000 (2 mg/ml). The optical density of the eluent was monitored at 280 nm.

4. Characterisation of solute behaviour

From the chromatograph obtained following elution of solutes from a column, the elution volume (V_e) of a given solute may be obtained. This parameter varies with the total volume of the packed bed (V_t). The void volume (V_o) is the elution volume of molecules that are excluded from the pores of the gel and hence are only distributed within the mobile phase, this parameter also varies with V_t.

Thus the elution position of a solute is best described by a coefficient (K_{av}) which relates the elution volume to the fraction of the stationary gel volume which is available for diffusion.

$$\text{i.e. } K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

It has been found that for a series of compounds of similar molecular shape, an essentially linear relationship exists between their K_{av} values and the logarithm of the molecular weight. Thus a

calibration curve using solutes of known molecular weight can be used to estimate the molecular weight of an unknown. Values for K_{av} thus vary between 0 (when $V_e = V_o$) and 1 (when $V_e = V_t$). However, non-ideal behaviour of solutes may result in values for K_{av} of less than zero or greater than one.

The following experimental conditions were employed:

Parameter	Sephadex G10	Sephadex G15
Column length (cm)	55.4	59.3
Column volume (ml)	39.6	42.3
Maximum load (ml)	2.0	2.1
Flow rate (ml/hr)	6.3	5.4
Eluent 1	Distilled water	
Eluent 2	Ammonium acetate 50 mmol/l, pH7.0	

The following peptides were used to calibrate the size exclusion column:

Standards	Molecular weight	
Bovine serum albumin	69,000	(V_o)
Bradykinin	1,241	
Bromophenol blue	670	
Gly-Gly-Tyr-Arg	452	
Tyr-Gly-Gly	295	
L-Tyrosine amide	180	

2.5.5 High performance liquid chromatography

Liquid/solid chromatography is a form of adsorption chromatography. A solute is dissolved in the mobile phase (solvent) and passed over an insoluble matrix. Separation is effected by the relative affinities of different solutes for the stationary phase. High performance liquid chromatography (HPLC) is characterised by

highly resolutive separation achieved by the use of uniform microparticulate (5-10 um) chromatographic supports. This implies both a greater area per unit volume for separation and the high perfusion pressure (30-250 bar), as compared with macroparticulate separation.

1. Choice of adsorbent

Most of the types of separations performed under near atmospheric pressure can also be effected by HPLC. Although most early HPLC separations were normal phase, ie a polar microparticulate silica support and use of non-polar eluents such as hexane, most current separations (60%) utilise a non-polar support and polar eluents and are hence termed reverse phase. While octadecyl (ODS; C_{18}) supports are most useful for separation of peptides with 15 residues or less, short chain (C_8, alkylphenyl or alkylnitrile) phases are used for larger peptides.

Reverse phase HPLC (RP-HPLC) has been used extensively for the purification of components of peptide and protein mixtures whether from tissue extracts (Byfield et al. 1976, Morris et al. 1980) or mixtures of peptides (e.g. Monch & Dehnen 1977, Tonnaer et al. 1980, Meek & Rosetti 1981, Browne et al. 1982, Geiger et al. 1982).

HPLC separations in this study were performed using a C_{18} support (Zorbax ODS, Du Pont) in both 4.6 mm x 30 cm (analytical) and 8 mm x 50 cm (semi-preparative) columns.

2. Choice of solvent

Since the support used is reverse phase, this implies aqueous buffers and development with organic solvents such as methanol, acetonitrile or isopropanol. Previous considerations of volatility of eluent and transparency to detection also apply.

Development of the chromatograph may take the form of gradient

or echelon elution. Echelon elution has the advantage of cheapness and simplicity, the steps are readily reproducible. This technique is most useful when examining samples containing compounds of widely differing polarities. It has the disadvantages of detection disturbance and inflexibility in optimising separations. Gradient elution has the advantage of flexibility and minimal disturbance to detection of the elution profile. Simple gradient formers using linked reservoirs are very problematic as the two solvents are likely to be of widely differing densities and viscosities. The problems are further compounded by the high flow rates required for elution. Gradient formation using time proportion or twin pump systems is much more reliable and reproducible. The principal disadvantage of this system is the cost. The implications of hold-up volumes on the resultant composition profile may limit the maximum effective rate of eluent composition change.

When developing the chromatographic system used in this study, gradient forming ability was not available and separation was effected by use of echelon elution, performed on a Waters M6000A solvent delivery system. The eluents used were 50 mmol/l ammonium acetate pH 7.0, at a flow rate of 1.0 ml/min and increments of 20%, 40%, 70% and 100% methanol in ammonium acetate solution. Initial experiments used acetic acid, 0.1% pH 3.5 as primary solvent. In an attempt to reduce loss of bioactivity during separation, the pH of the solution was raised to pH 7.0,which renders peptides more polar and hence reduces their elution time. Use of phosphate buffers was avoided, as previous work (Schlievert et al. 1976a, 1977) had indicated that these ions were inhibitory to the low molecular weight antibacterial component of amniotic fluid and, unlike ammonium acetate, were non-volatile on lyophilisation.

The alcoholic eluents possessed intrinsic antibacterial activity but were volatile. Therefore, the methanol in each fraction was evaporated as far as possible under a jet of nitrogen gas. The residuum was freeze dried and antibacterial activity assessed.

Gradient forming facilities became available toward the end of the study, by use of a Spectra-Physics 8700 Ternary solvent system. The column was equilibrated in ammonium acetate buffer prior to use at a flow rate of 1.0 ml/min and the sample injected. A gradient was simultaneously applied, the concentration of methanol changed linearly from 0 to 100% over 50 minutes (i.e. +2%/min). At the end of the gradient, the column was washed for 10 minutes with 100% methanol. The starting conditions were then re-established.

2.5.6 Ion exchange chromatography

The basic principle of ion exchange is that separation is achieved on the basis of the difference in charges carried by the solute molecules. These differences may be very small and hence ion exchange chromatography can be a technique of very high resolving power.

1. Choice of gel matrix

An ion exchanger consists of an insoluble matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counter-ions which may be exchanged with other ions of the same charge. Thus positively charged resins have mobile anions available for exchange, while negatively charged matrices may exchange positively charged counter ions.

The choice of matrix depends on:

a) the stability of the solute in solution

b) the molecular size of the solute

c) the specific requirements of the separation.

Both of the studies of Schlievert et al. (1976c) and Sachs & Stern (1979) used the weak anion exchanger diethyl aminoethyl (DEAE) A-25 ion-exchange resin. DEAE A-25 was investigated together with its cation exchange equivalent, carboxymethyl (CM) C-25 resin. The strong cation exchange resin, sulphopropyl (SP) C-25, was also studied.

The test-tube method was used to determine choice of matrix and starting pH. Ion exchanger (0.1 g), in which the counter ions had been replaced by ammonium acetate, was added to a series of tubes. The gel in each tube was changed to a different pH by washing with 0.5 mol/l buffer and equilibrated in 50 mmol/l buffer. DEAE A-25 was prepared with a pH range of 5 to 9 with 0.5 pH unit intervals between tubes. CM C-25 and SP C-25 were prepared with a pH range of 4 to 8 with 1.0 pH unit intervals. An aliquot of acid alcohol extract was added to each tube and the gel mixed for 10 minutes. The gel was allowed to settle and the supernatant assayed for antibacterial activity. Weak ion exchangers (DEAE- and CM-types) are preferentially used for separation of labile materials as milder conditions are required for elution. Strong ion exchangers (QAE- and SP-types) are used for separation of weakly ionising substances and those which require extremes of pH for ionisation.

2. Choice of column

Ion exchange chromatography is usually run on short broad columns, as separation is determined by varying elution conditions. Previous workers have used columns either 1.5 by 50 cm (Schlievert et al. 1976c) or 2.6 by 20 cm (Sachs & Stern 1979).

3. Choice of eluent

Similar arguments, for the use of ammonium acetate as eluting buffer, apply as for use with size exclusion chromatography. In addition, elution pH was determined by the use of the particular

matrix. Anion exchange requires the starting buffer to be alkaline and elution effected by decreasing pH gradient or increasing ionic strength. Cation exchange requires an acidic loading buffer and elution by increasing pH or ionic strength gradients. The exchangers are supplied with either sodium or chloride counter-ions, hence for use with ammonium acetate the counter ion was replaced by suspending the exchanger in 1.0 mol/l ammonium acetate and washing several times before finally washing with starting buffer. As with size exclusion chromatography slower flow rates give better resolution, a linear flow rate of 10 cm/hr is recommended. The total volume of eluent in a gradient should be approximately five times the bed volume, longer or shorter gradients are usually detrimental to separation (Novotny 1971).

4. Experimental conditions

The following experimental conditions applied. A column of 2.6 cm diameter, filled with DEAE A-25 in slurry form, gave a final bed height of 19.5 cm and hence a bed volume of 104 ml.

The column was equilibrated at pH 7.5 with 10 mmol/l ammonium acetate buffer. The sample was loaded and washed onto the column in 20 ml of buffer. A 500 ml gradient extending from 10 mmol/l ammonium acetate, pH 7.5 to 1000 mmol/l was used. A final 50 ml wash of 1.0 mol/l sodium chloride was also applied. The flow rate used was 25 ml/hr (= 4.7 cm/hr) and fraction size was 10 ml. Fractions were lyophilised following collection, resolved in 10 µmol/l zinc chloride and assessed for antibacterial activity to B. subtilis.

2.6 Characterisation techniques

2.6.1 Affinity chromatography

This is a form of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a biospecific ligand, immobilised on an inert chromatographic matrix. A bead-formed agarose gel forms an open-pored matrix which exhibits very low non-specific adsorption. Hydroxyl groups on the sugar residues can be easily derivatised for covalent attachment of ligand.

The selected ligand should exhibit specific and reversible binding affinity for the substance to be purified. It should also have chemically modifiable groups which allow for attachment to the matrix without inactivating the binding capacity. Group specific adsorbents have affinity for a group of related substances, rather than for a specific substance.

Adsorbent ligand	Specificity
Con A-Sepharose 4B	Glucose, Mannose
Lentil lectin-Sepharose 4B	Similar to Con A-Sepharose, but lower affinity and resistant to deoxycholate
Wheat germ lectin-Sepharose 6MB	N-acetylglucosamine
Soybean lectin-Sepharose	N-acetylgalactosamine

Columns containing 5 ml lectin-sepharose were washed with 10 volumes of starting buffer (20 mmol/l Tris-HCl, 500 mmol/l NaCl, pH 7.4) to remove preservative. The sample was loaded, washed into the column, and incubated at 4^{o}C for 1 hour. The column was then washed with 4 volumes of starting buffer, followed by 3 volumes of elution buffer 1 (500 mmol/l sugar, dissolved in starting buffer) and 3

volumes of elution buffer 2 (starting buffer adjusted to pH 3.5). All fractions were monitored for antibacterial activity.

Washing with starting buffer removed unbound material, while buffer 1 desorbed compounds with the specific sugar moiety of interest. Tightly (non-specifically) bound substances were eluted with buffer 2 and the gels stored in starting buffer adjusted to pH 6 and containing Merthiolate (0.01%), manganese chloride (1 mmol/l) and calcium chloride (1mmol/l).

2.6.2 Thin layer chromatography

1. Amino acid chromatography

One- and two- dimensional thin layer chromatography (TLC) was performed using the methods of Ersser (1970). Samples, both hydrolysed and non-hydrolysed, were dissolved in 10% aqueous isopropyl alcohol and, together with standard amino acids (0.4 - 2 nmol), loaded onto cellulose F1440 TLC plates (Merck). For one-dimensional chromatography, the prepared plate was developed twice in solvent 1 (Butanol:Acetone:Acetic acid:Water, 35:35:10:20, by volume), drying the plate between runs. For two-dimensional chromatography, the plate was developed first in solvent 2 (2-methylpropan-2-ol:Butanone:Ammonia (35%):Water, 30:50:7:23 by volume), the plate was then dried and developed twice in the second direction, in solvent 1, as before.

After development, the plates were dried and the amino acids visualised with 0.5% (w/v) ninhydrin in acetone and heating at 50°C.

For hydrolysis, the samples were dissolved in 6 mol/l hydrochloric acid and heated to 110°C for 16 hours. The hydrochloric acid was then removed by volatilisation at 30°C under a stream of nitrogen. The hydrolysed samples were then dissolved in

10% aqueous isopropanol and loaded onto TLC plates as previously stated.

2. Sugar chromatography

One dimensional ascending chromatography was performed using the methods of Menzies & Mount (1975) for neutral sugars and those of Hunt (1972) for amino sugars.

a) Neutral sugars

Samples were desalted (using zerolit resin) and loaded onto F1500 silica gels. Standard aldose and ketose sugars were also applied (2 - 10 nmol). The plates were dried and developed twice (in ethyl acetate:pyridine:acetic acid:water, 60:30:5:15, by volume) with thorough drying between runs. After chromatography, the sugars were visualised using a single-stage reagent (p-aminobenzoic acid, 10% in methanol:o-phosphoric acid:methanol, 14:4:82, by volume).After drying the colour reaction was developed at 130^{o}C.

b) Amino sugars

Amino sugars in complexed form were released by acid hydrolysis (2 mol/l hydrochloric acid, 100^{o}C for 3 hours, under nitrogen). The acid was removed and any contaminating amino acids removed by treatment with zerolit resin. The samples and standards (0.4 - 2 nmol) were then loaded onto F1440 Cellulose gels and developed twice (in n-butanol:pyridine:water, 6:4:3, by volume), drying thoroughly between runs.

The amino sugars were visualised using Elson-Morgan reagent (aqueous potassium hydroxide (25%) : ethanol (95%) : ethanolic acetylacetone (10%), 2:8:10, by volume, followed by heating (110^{o}C) for 5 minutes. The chromatograph was then dipped in a solution of p-dimethylamino-benzaldehyde, 10% in conc. hydrochloric acid:ethanol (95%), 1:1, by volume.

2.6.3 Chelation studies

Samples of amniotic fluid were tested for antibacterial activity. Each sample was divided into seven aliquots. The first from each sample was incubated at 4°C for 24 hours, 3 subsquent aliquots were treated with (a) ethylenediamine tetra-acetic acid (EDTA), 5 mmol/l; or (b) 1,10-phenanthroline (OPA), 0.5 mmol/l; or (c) diphenylthiocarbazone ('Dithizone'), 0.5 mmol/l; and then incubated at 4°C for 24 hours. Three further aliquots were first incubated for 24 hours and then treated as at (a), (b) and (c) respectively. All aliquots were then centrifuged (2000 g, 15 min), ultrafiltered (Amicon YM2 membrane, 50 psi) and the residue reconstituted to the original volume with phosphate-buffered saline (PBS), pH 7.4. The chelating agents were removed: EDTA and OPA by ultrafiltration and Dithizone by centrifugation. The antibacterial activity of each aliquot (1 just incubated; 3 treated with chelating agents, incubated and the agents removed; and 3 incubated, treated with chelating agents, and the agents removed) was then determined.

Each of the seven aliquots was then further subdivided and again incubated for 24 hours at 4°C, in the absence or presence of one of the following divalent cations (added as the chloride): magnesium and calcium, both 5 mmol/l; manganese, iron, cobalt, copper and zinc, all 15 umol/l. Controls were incubated and then treated with one of the cations above. The antibacterial activity of each sample was then determined.

For chelation studies, each acid alcohol extract was divided into 7 aliquots, and treated with chelating agents as described above. Following incubation, all aliquots were assayed for antibacterial activity against B. subtilis. The aliquots were then

sub-divided and incubated in the absence or presence of divalent cations. The antibacterial activity of each sample was then determined.

Further samples of acid alcohol extracts were also exposed to dithizone as above. Following removal of the chelating agent, the extract was incubated with 10 nmol/l radioactive zinc (^{65} Zn) and fractionated by echelon elution HPLC. The eluent was collected in 0.5 ml fractions and monitored not only for antibacterial activity but also for the presence of radio-zinc.

Although ^{65}Zn is prinicipally a high energy gamma ray emitter, it also emits B-particles. Therefore, radioactivity was determined in an Intertechnique SL3000 Liquid Scintillation counter, using Unisolve scintillant. Activity was expressed in counts-per-minute (cpm) determined by external standard radiocounting coupled to quench correction by computation from a preprogrammed quench curve.

2.6.4 Enzymology

Samples (0.5 ml) of acid alcohol extracts of lyophilised ultrafiltrate of amniotic fluid were exposed to 0.5 ml volumes of enzyme solution, which were prepared as detailed below. After exposure, the sample was chilled to 0°C and ultrafiltered in an Amicon model 52 cell. The retentate was twice resuspended in reaction buffer and filtered. The ultrafiltrates were combined and lyophilised before testing for antibacterial activity, together with like-treated controls. Controls were incubated under the same conditions as the test solutions except that the enzyme solution was added immediately prior to chilling to ice temperature. Reaction buffer - ammonium acetate, 100 mmol/l, pH 7.4.

1. Trypsin (EC 3.4.21.4)

Trypsin solution (0.4 mg enzyme/ml) was prepared in reaction buffer supplemented with calcium chloride 40 mmol/l. The extracts were exposed to the enzyme for 4 hours at 25°C.

2. Chymotrypsin (EC 3.4.21.1)

The enzyme solution was prepared in reaction buffer at 40 mg/ml. The incubation conditions were 4 hours at 25°C.

3. Carboxypeptidase A (EC 3.4.12.2)

Carboxypeptidase was prepared at 0.4 mg/ml in reaction buffer supplemented with lithium chloride (10% w/v). The samples were incubated in the presence of the enzyme for 4 hours at 25°C.

4. Staphylococcal protease (EC 3.4.21.19)

The enzyme was prepared at 0.4 mg/ml in reaction buffer, which had been adjusted to pH 4.0. The samples were digested for 4 hours at 37°C.

5. Amylase (EC 3.2.1.1)

The enzyme solution was prepared at a concentration of 0.1

mg/ml in phosphate buffer (20 mmol/l, pH 7.0: sodium chloride 50 mmol/l). The samples were exposed to the enzyme for 4 hours at 37°C.

6. Glycoprotein-B-D-N-acetylglucosamine transferase (EC 2.4.1.51)

The glycoprotease was prepared in reaction buffer supplemented with uridine diphosphate (10 umol/l). Samples were exposed to the enzyme for 4 hours at 37°C.

2.6.5 Electrophoretic analysis

Polyacrylamide gel electrophoresis was performed by the method of Swank & Munkres (1971), and carried out by Dr J.F. Guest. The gels were stained using a commercial silver staining procedure (Bio-Rad Silver Stain Kit). Following staining, the gel lanes were excised from the polyacrylamide slab and cut into 0.5 cm slices. Each slice was destained separately, by soaking in 10% (w/v) X-ray liquid fixer (Kodax FX40), washed in deionised water and stored at -20°C.

Gel slices were homogenised in acid alcohol extraction medium and incubated for 24 hours at 4°C. The extract was evaporated under nitrogen, dried *in vacuo*, and assayed for antibacterial activity.

Table 5. Possible tetra-peptide sequences based on the composition Gln_2, Pro_1, Gly_1.

1	Gln-Gln-Pro-Gly
2	Gln-Pro-Gln-Gly
3	Pro-Gln-Gln-Gly
4	Gln-Gln-Gly-Pro
5	Gln-Gly-Gln-Pro
6	Gly-Gln-Gln-Pro
7	Pro-Gly-Gln-Gln
8	Gly-Pro-Gln-Gln
9	Gln-Pro-Gly-Gln
10	Pro-Gln-Gly-Gln
11	Gln-Gly-Pro-Gln
12	Gly-Gln-Pro-Gln

2.7 Peptide synthesis

2.7.1 Introduction

The results of the preceeding experiments all suggested that the low molecular weight antibacterial component extracted from amniotic fluid was peptide in nature. In addition, fast atom bombardment mass spectroscopy (FAB-MS) on a purified fraction showed the presence of a compound with a mass/charge ratio of 429 which could correspond to a peptide of a composition Gln_2, Pro_1, Gly_1 (Morris et al. 1983, personal communication). The alternative possibility of a compound with a composition of Glu_1, Gln_1, Pro_1, Gly_1 with the carboxy function of the C-terminus converted into the amide form (e.g. Glu-Gln-Pro-Gly-NH_2) was negated by the absence of a blocked C-terminus as evidenced by the enzyme studies conducted previously.

Thus given a composition of Gln_2, Pro_1, Gly_1, there are twelve permutations of these amino acids to form a tetrapeptide chain (Table 5).

Essentially peptide synthesis consists of 3 steps. First, those carboxyl and amino groups not participating in the reaction are chemically blocked. Secondly, the free amino and carboxyl functions are linked. Thirdly, the blocking groups are removed. Amino acids which contain side chains that may react during the condensation reaction, must have such side chains blocked.

2.7.2 Peptide synthesis methods

Peptides were synthesised by use of the mixed anhydride method (Weiland et al. 1950, Weiland & Sehring 1950). Synthesis was started using protected amino acids. The amino group of the carboxyl component of the reaction was protected with a t-butyl-oxycarbonyl (Boc-) grouping while the carboxyl group of the amino component was

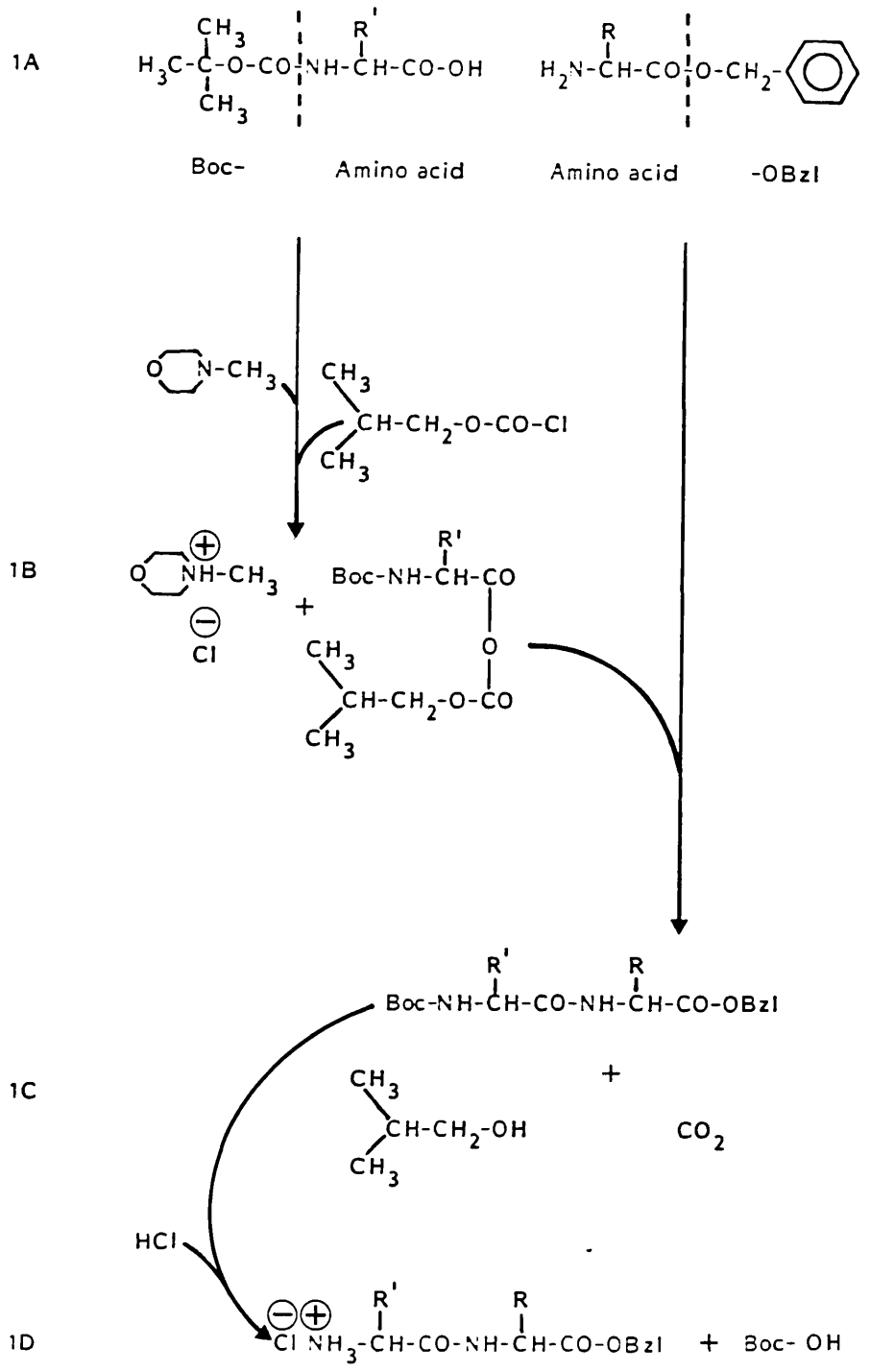


Fig. 1. Route of synthesis of a dipeptide from protected amino-acid precursors, by the mixed anhydride method.

protected with a benzyl ester (-OBzl), Fig. 1A.

The mixed anhydride was prepared by the addition of a carbonic acid-half ester-half chloride (isobutyl chloroformate), as proposed by Vaughan & Osato (1952), to a cooled solution (-20°C) of the Boc-protected amino acid together with the tertiary base, N-methylmorpholine, in an organic solvent (ethyl acetate). The formation of the mixed anhydride was practically instantaneous as indicated by the immediate precipitation of N-methylmorpholine hydrochloride, Fig. 1B. The mixed anhydride so formed was used in the acylating step without prior purification or isolation. The advantage of this approach lies in the lack of stability of the carbonic acid half esters liberated during acylation. These immediately decomposed to the corresponding alcohol and carbon dioxide, Fig. 1C.

The reaction was driven by an excess of carbonyl components. Thus on completion, the reaction mixture contained protected dipeptide, surplus Boc-anhydride, isobutanol and N-methylmorpholine hydrochloride. Work up into aqueous solution removed the non-perpetuant materials.

The resultant dipeptide was deprotected at the N-terminus using 4 mol/l hydrogen chloride in dioxane, Fig. 1D. The nascent amino group was then coupled to another amino acid. The process was repeated to form the tetra-peptide product.

The above method was used for synthesis of tetra peptides 1 to 6. For peptides 7 to 12, the initial amino component, Glutamine O-benzyl ester, was not available. Boc-Gln-OBzl was synthesised from Boc-Gln-OH using the method of Kamijo et al. (1984) and its structure confirmed by nuclear magnetic resonance spectroscopy. This was then deprotected in HCl/dioxane to give the amine salt (NH_2-Gln-OBzl HCl) which was then used as previously described.

The reactions were followed by thin layer chromatography. Starting materials and products of each coupling were spotted onto cellulose F1440 TLC plates and the chromatograph developed in chloroform:methanol:acetic acid (17:2:1). The amino acids and peptides were visualised with ninhydrin and heating. Deprotection of the final peptide product was performed using hydrogen bromide in acetic acid (45% w/v).

Antibacterial activity of each peptide was assessed both with and without prior incubation (24 hours,4^{o}C) in the presence of zinc chloride (10 µmol/l). Each peptide was prepared in serial ten-fold dilutions ranging from 10 mmol/l to 1 nmol/l. Activity was also assessed following purification by gradient elution reverse phase HPLC (section 2.5.5).

2.8 Statistical analysis of data

Analysis of data was performed on the PE3220 computer in the computer centre of the Royal Postgraduate Medical School, using the 'Minitab' statistical package. Relationships between nominal scale variables were examined using chi-square analysis with either Yates' continuity correction or Fisher's exact test where appropriate. (Cochran 1954).

Ordinal scale variables were compared using Students' t-test. Groups with positively skewed distributions were subjected to logarithmic transformations which were then used in statistical calculations. Correlation of variables were assessed using the Pearson product moment correlation test and significance levels derived using the standard error estimate.

All necessary underlying statistical assumptions were verified prior to application of statistical analysis. Summary statistics of ordinal scale variables are presented as mean $\pm$ 1 standard deviation (1 s.d.) or mean $\pm$ 1 standard error of the mean (1 s.e.). Results are reported as statistically significant if $P < 0.05$, two-tailed test.

CHAPTER THREE

Antibacterial activity of human amniotic fluid

Most studies on the antibacterial activity of amniotic fluid have focussed on the incidence of fluids inhibitory to the indicator organism(s) under examination. The effects of various routine contaminants of amniotic fluid, on expression of activity have been inadequately reported. It was intended that the experiments of this chapter should examine, in a quantifiable manner, the antibacterial activity of amniotic fluid against the indicator organism. Further, it was proposed that the effects of local environmental contaminants should be examined, and quantified where possible.

Table 6. Antibacterial activity (mean ± 1 s.d.) of amniotic fluid, as assessed by Plate Diffusion and Tube titration.

Amniotic Fluid	Plate diffusion (units/ml)				Tube titration (MIC)			
	1	2	3	m	1	2	3	m
1	7.6	6.5	8.0	7.4 ± 0.8	4	8	2	5 ± 3
2	5.1	5.8	7.6	6.2 ± 1.3	8	8	4	7 ± 2
3	2.7	2.6	5.3	3.5 ± 1.5	2	1	8	4 ± 4
4	10.2	15.1	12.0	12.4 ± 2.5	4	16	8	9 ± 6
5	3.5	2.5	2.6	2.9 ± 0.6	4	8	2	5 ± 3
6	21.6	22.3	18.7	20.9 ± 1.9	8	32	32	24 ±14

The two standard techniques for determining the antibacterial activity of solutions were compared for use in the study for the assessment of the antibacterial activity of amniotic fluid.

Table 7. Antibacterial activity (units/ml) of amniotic fluid, assessed in Plain and Siliconised Plates.

Amniotic fluid	Plain plates	Siliconised plates
1	32.9	24.8
2	12.0	10.3
3	4.0	10.0
4	3.5	3.5
5	20.6	22.4
6	2.5	2.5

The antibacterial activity of amniotic fluid was assessed both in plain plates and in those plates which had been rendered inert by siliconisation.

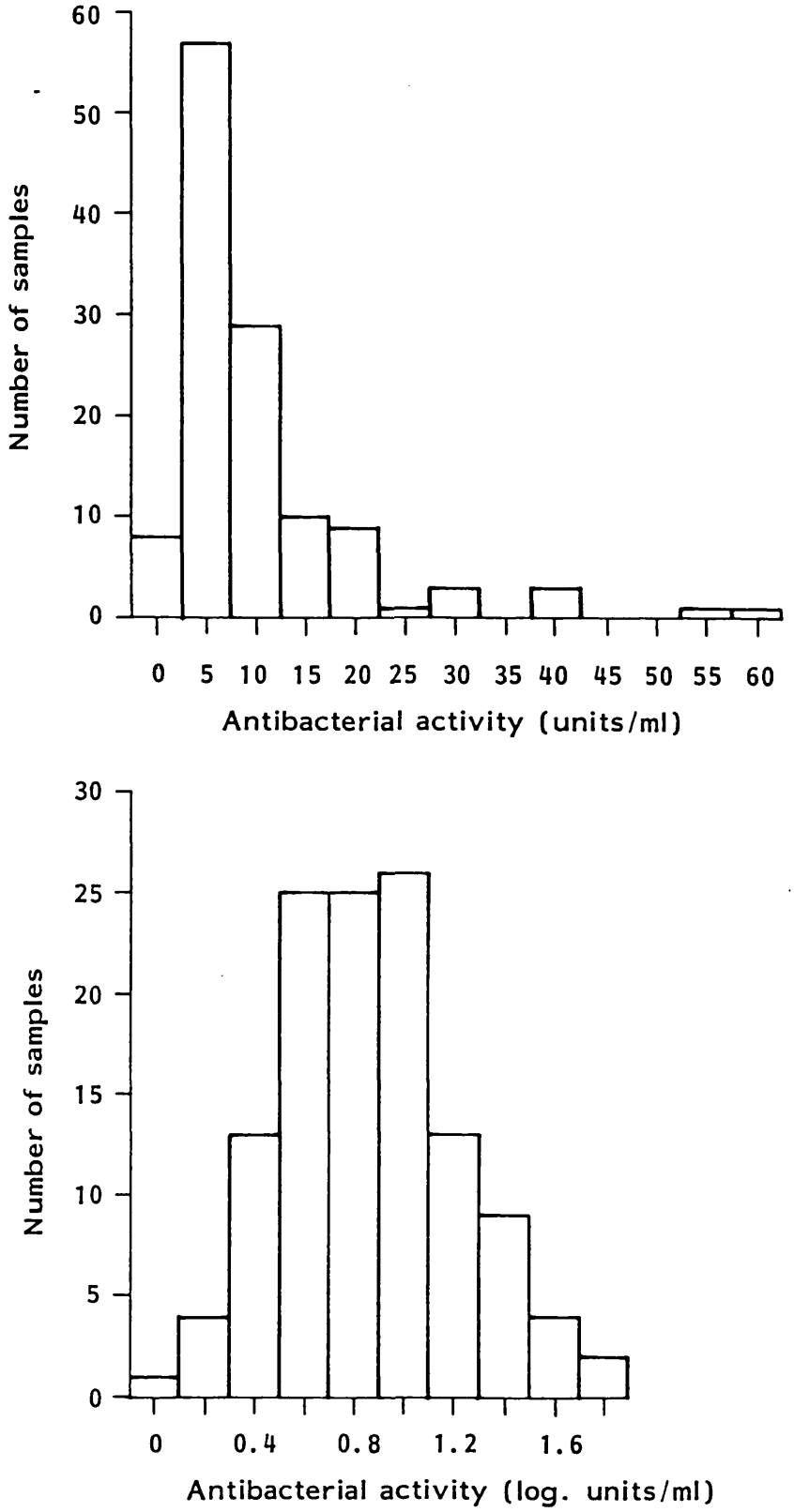


Fig. 2. The frequency distribution of antibacterial activities of human amniotic fluids, displayed in arithmetic and geometric forms.

3.1 Antibacterial activity of whole amniotic fluid

3.1.1 Selection of assay technique

The two methods of assessing antibacterial activity of solutions (the tube titration and plate diffusion assays) were compared for use in detection of activity against Bacillus subtilis in samples of whole amniotic fluid. Six samples of amniotic fluid were assessed three times, in duplicate. The results are tabulated opposite (Table 6).

The agar plate diffusion assay gave more consistent results than the tube titration method; the coefficients of variation in the former, ranged from 9 to 43%, while those of the latter, ranged from 35 to 105%. There was no apparent difference in the sensitivity of the two assays. Accordingly, it was decided that the plate diffusion assay should be adopted for routine use.

β-lysin adheres strongly to plastics, which may interfere with assessments of antibacterial activity (Donaldson 1977). Therefore, comparisons of antibacterial activity were made using plain plates and those rendered inert by siliconisation. The results are shown in Table 7. Analysis of the results, using a paired t-test, did not reveal any difference between the two types of assay plates. Therefore, untreated plates were used in this study.

3.1.2 Activity of amniotic fluid against Bacillus subtilis

Antibacterial activity was assessed by the plate diffusion method in 122 samples of amniotic fluid, which were free from contamination. Antibacterial activity was defined as the reciprocal of the dilution which would be expected to reduce the activity of the sample to zero. It was present in all the amniotic fluids obtained near term. Values for activities had a positively-skewed, non-normal distribution (Fig. 2a). Transformation of the data, by taking the common logarithm (log 10), resulted in a normal distribution ($P < 0.01$, Fig. 2b).

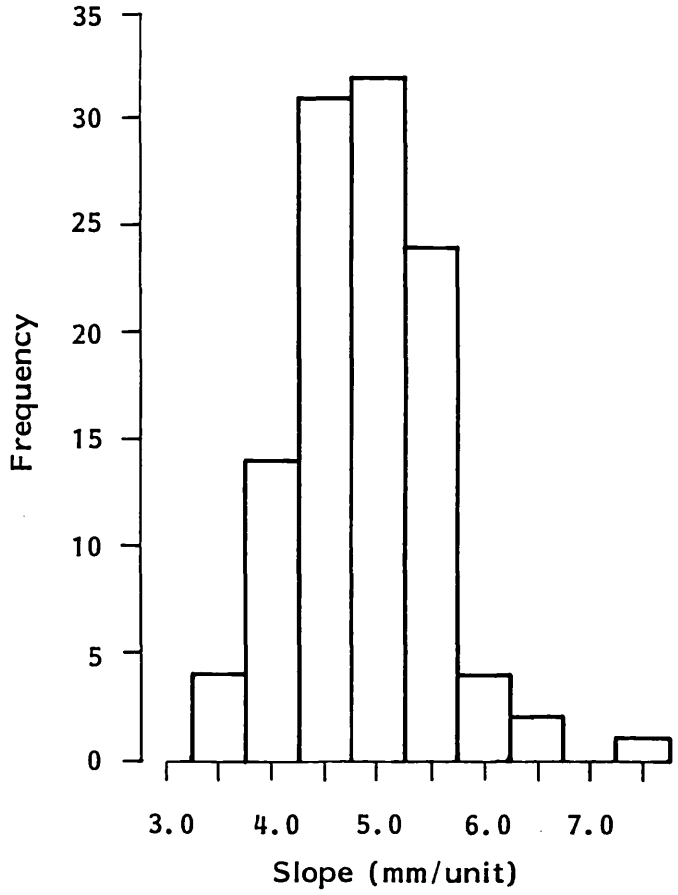


Fig. 3. The distribution of slopes derived from the bacterial growth inhibition curves of 122 samples of whole amniotic fluid.

and hence this was used in subsequent statistical calculations. The arithmetic mean (±1 s.d.) of the antibacterial activity was 10.2 ± 10.1 units/ml giving a standard deviation range of 0.1 to 20.3 units/ml. The mean (±1 s.d.) of the transformed data was 0.859 ± 0.353 log. units/ml which produces a geometric mean of 7.2 units/ml with a standard deviation range of 3.2 to 16.3 units/ml.

The other datum generated, by the analysis of the plate diffusion assay is that of the slope of the dose response curve from the following equation:

$$y = a + bx \qquad 1.$$

where y is the diameter of the zone of inhibition (in mm),

x is the activity of the solution (in units/ml),

a and b are constants,

a is the diameter of the well (in mm)

and b is the slope of the growth inhibition curve (in mm/unit)

Since logarithmic transformation of the antibacterial activity values produced a normal distribution, equation 1. becomes:

$$y = a + b.\log(x) \qquad 2.$$

The slopes of the growth inhibition curves were normally distributed with a mean of 4.9 ± 0.6 mm/unit (Fig. 3). The slope of the outlying point may have been strongly influenced by the presence of low molecular weight antibacterial component (section 4.1.2) in the amniotic fluid. The slopes of the bacterial growth inhibition curves were therefore divided into 2 groups on the basis of the presence or absence of detectable activity due to the low molecular weight component. The mean slope of the inhibition curves in those samples without detectable low molecular weight activity was 4.74 ± 0.54 mm/unit (n = 73), as compared with 4.95 ± 0.65 mm/unit (n = 29)

for those amniotic fluids with inhibitory ultrafiltrates. This difference was not significant ($P = 0.097$), however it is suggestive that, in those amniotic fluids which possess detectable low molecular weight activity, this component may contribute to the overall antibacterial activity of the sample.

It may not be appropriate to make firm conclusions on the basis of the slope of the antibacterial assay. It seems likely that the relative proportions of the various antibacterial factors of amniotic fluid, may vary from sample to sample. It is possible, therefore, that the variability seen (in Fig. 3) is a reflection of differences in the contributions of these factors to the overall activity of individual samples.

Additionally, antibacterial assay plates should be standardised. A standard dose of an antibiotic, such as penicillin, is randomly allocated across the assay plate. Inhibition zones produced by the test substance may be corrected for differences seen in zones produced by the standard. Such a technique was not employed in this study, which may thus have contributed to the variation in the slopes of the antibacterial assay.

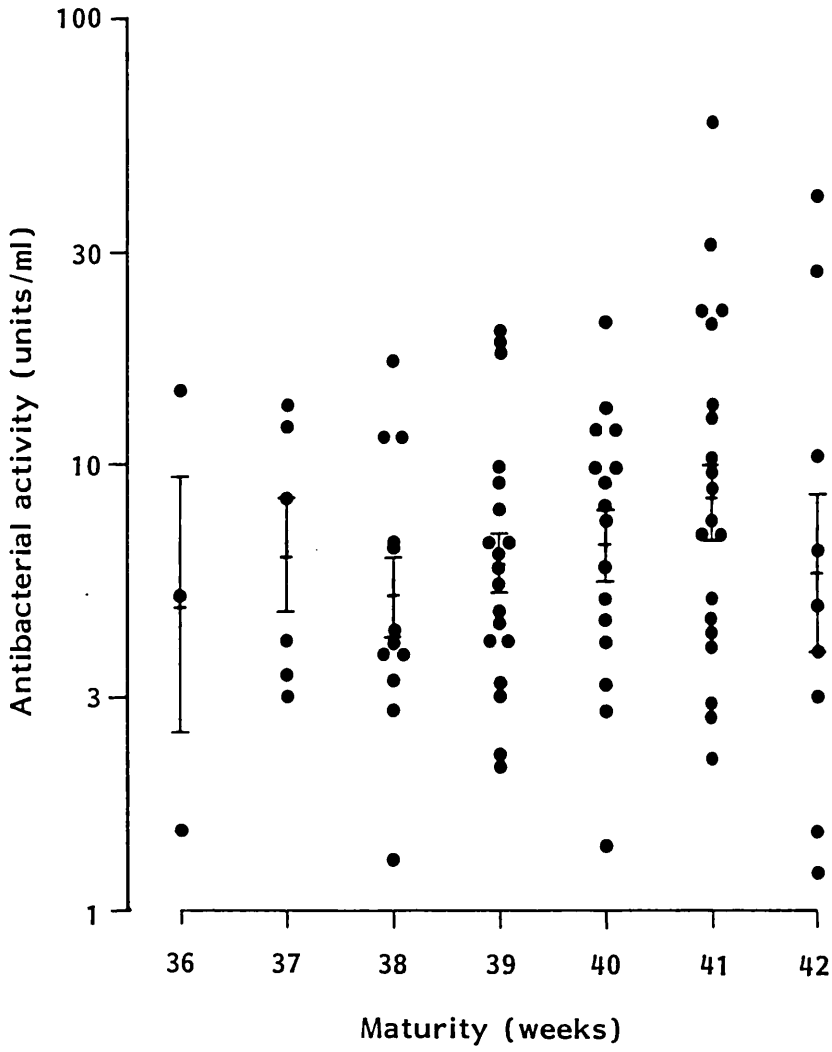


Fig. 4. Antibacterial activity (units/ml; mean ± s.e.) of amniotic fluid collected between 36 and 42 weeks of pregnancy.

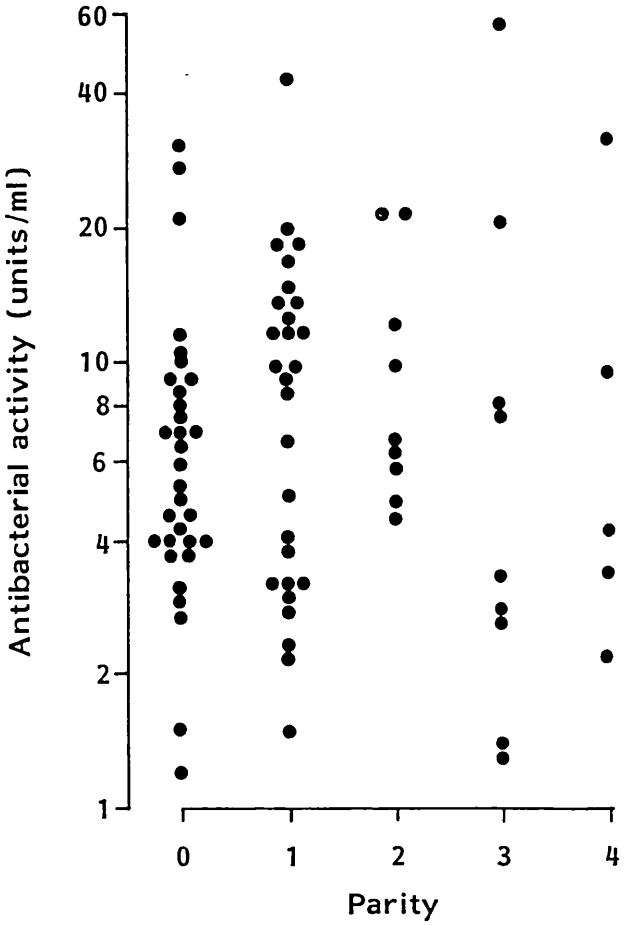


Fig. 5. The distribution of antibacterial activities of amniotic fluids obtained from both nulliparae and multiparae.

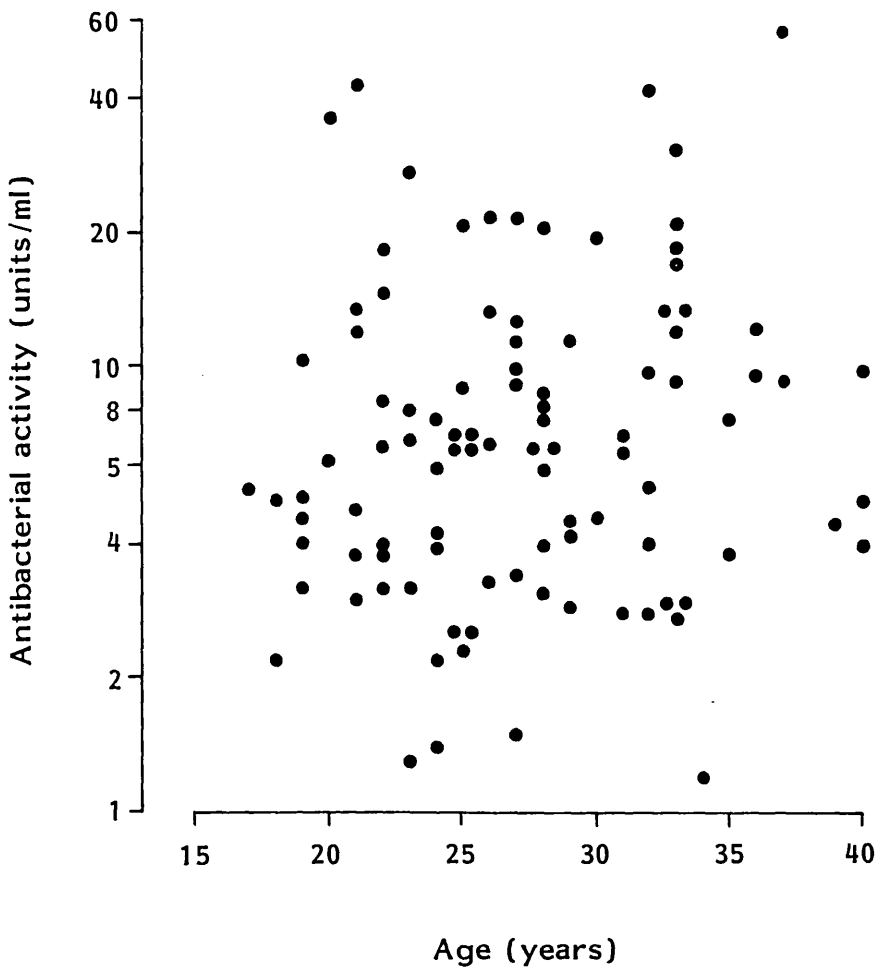


Fig. 6. The distribution of antibacterial activity in amniotic fluid with relation to maternal age.

3.2 Relation of antibacterial activity of amniotic fluid to epidemiological factors

3.2.1 Maturity

Data on the maturity of the pregnancy at the time of collection of amniotic fluid was available from 89 patients. The amniotic fluids were collected mainly between 36 and 42 weeks of pregnancy. Between these limits, there was no relation between antibacterial activity and the maturity of pregnancy (Fig. 4). Antibacterial activity was also assessed in 9 samples of amniotic fluid obtained between 16 and 32 weeks of pregnancy. Three samples collected at week 16 were all inactive. One sample collected at 23 weeks of pregnancy that was inactive on initial testing, was concentrated 20-fold and revealed antibacterial activity equivalent to 0.2 units/ml in the neat amniotic fluid. Three of the five samples collected between 27 and 32 weeks of pregnancy possessed detectable activity against *B. subtilis*.

3.2.2 Parity

Data on the parity of the patients, from whom the samples were collected, was available in 89 cases. The parity of these patients ranged from 0 to 4. There was no relation between antibacterial activity of amniotic fluid sample and the parity of the patient (Fig. 5).

3.2.3 Age

The age of the patient at the time of collection of the amniotic fluid sample, was also recorded in 99 cases. The ages of these patients ranged from 17 to 40 years. There was no relation between antibacterial activity of the amniotic fluid and the age of the patients (Fig. 6).

3.2.4 Indication for induction of labour

Information concerning the indication(s) for induction of labour was obtained in 82 cases. In some instances, secondary indications existed in addition to the principal indication. There was no association of activity with a particular indication. Twenty-one patients were induced due to post-maturity (duration of pregnancy exceeding 40 weeks + 10 days), while 17 patients were induced due to cardiovascular disturbances which ranged from elevated blood pressure to moderate pre-eclampsia. Amniotic fluid was collected from 10 patients whose membranes were ruptured to expedite the progress of labour.

3.2.5 Source of amniotic fluid

Amniotic fluid was obtained from patients in two geographic locations. Of the 122 samples of amniotic fluid discussed earlier, 98 samples were obtained from Hammersmith Hospital and 24 from Northwick Park Hospital. The geometric mean antibacterial activity of amniotic fluid from Hammersmith Hospital was 6.8 (3.0 - 15.6) units/ml as compared with 9.1 (4.4 - 18.9) units/ml found in amniotic fluid obtained from Northwick Park Hospital. This difference was not significant.

3.3 Studies of the effects of obvious contaminants of amniotic fluid

The work presented deals mainly with 'clean' specimens containing no obvious blood, meconium or 'Hibitane'. In order to assess the possible effects of various contaminants, a number of atypical specimens were examined, together with those samples containing vernix caseosa and sedimentation products.

3.3.1 Vernix caseosa

Vernix caseosa collected from amniotic fluid by centrifugation, emulsified and tested in the agar plate diffusion assay revealed no inhibitory activity to *B. subtilis*. Similarly, when emulsified in amniotic fluid and tested, vernix caseosa did not inhibit the expression of growth inhibitory activity of amniotic fluid. It was, however, removed routinely as its presence in amniotic fluid caused the ultrafiltration membranes to become blocked rapidly.

3.3.2 Sedimentation products

Sediments were obtained by centrifugation of amniotic fluids not contaminated with meconium, blood or antiseptic cream. They were collected, emulsified and tested in the agar plate diffusion assay and were found to be non-inhibitory to *B. subtilis*. Like vernix caseosa, there was no inhibition of antibacterial activity of amniotic fluid. However, this material also caused fouling of the ultrafiltration membranes and was removed routinely from amniotic fluid.

3.3.3 Blood-stained specimens

The extent of blood staining was graded from 'none' to 'trace' where flecks of blood were observed in the sediment on centrifugation, 'light' and 'heavy' when the amniotic fluid was red in colour and contained larger amounts of blood clot in the sediment.

Table 8. The effect of varying degrees of contamination with blood on the expression of antibacterial activity by amniotic fluid.

Degree of contamination	n	geometric mean (units/ml)	standard deviation (units /ml)	standard error (units/ml)
none	122	7.3	3.2 - 16.4	1.7 - 7.8
trace	3	7.6	2.5 - 23.1	4.0 - 14.8
light	6	15.9	8.5 - 29.8	12.3 - 20.6
heavy	5	23.4	9.8 - 55.8	15.9 - 34.6

The activity of amniotic fluid samples with either light or heavy contamination with blood was greater than that of uncontaminated samples ($P < 0.05$).

The results of the antibacterial activity analysis are shown in Table 8.

The mean antibacterial activity of specimens of amniotic fluid containing 'trace' amounts of blood was no different from that of clean amniotic fluid. The mean activity of specimens of amniotic fluid with 'light' or 'heavy' contamination with blood was significantly greater than that of clean amniotic fluid ($P < 0.05$, and $P < 0.05$, respectively). Additionally, the slope of the diffusion assay in heavily stained samples of amniotic fluid was 7.2 ± 0.54 mm/unit as compared with that of uncontaminated amniotic fluid (4.9 ± 0.62 mm/unit, $P < 0.001$). Thus blood-stained amniotic fluids were not used for further experimentation.

3.3.4 Meconium-stained samples

The 'degree' of contamination of amniotic fluid with meconium was not recorded, simply its presence or absence. All contaminated fluids had a distinctive green colour and produced a dense green sediment on centrifugation. Seven samples were tested for antibacterial activity to B. subtilis and were all inactive.

3.3.5 'Hibitane'-stained amniotic fluid

Gross contamination of samples of amniotic fluid examined soon after collection, was easily distinguished by the presence of creamy droplets dispersed throughout the sample. Such specimens were discarded. The antiseptic cream is, however, water-soluble. Heavily contaminated samples which had been stored for a period of time at $4^{o}C$ were visually indistinguishable from samples containing vernix caseosa. Thus stored samples (i.e. overnight collections and those from Northwick Park Hospital), together with those samples with suspected contamination were tested using the chlorhexidine assay (section 2.4.1). The limit of detection of this assay was 1.3 ug/ml

chlorhexidine acetate which was equivalent to 2 ug/ml 'Hibitane' (chlorhexidine gluconate). The detection limit of chlorhexidine acetate in the plate diffusion assay was also 1.3 ug/ml. Thus, if the concentration of chlorhexidine was below the limit of detection for the physicochemical method, then it did not interfere in the antibacterial assay. In addition, the slope of the bacterial growth inhibition curve of chlorhexidine, diluted in either sterile distilled water or amniotic fluid, was 9.3 $\pm$ 0.27 mm/unit ($n = 5$) as compared with that of amniotic fluid alone (4.9 $\pm$ 0.62 mm/unit, $P < 0.001$). Thus, samples contaminated with chlorhexidine were distinguishable from uncontaminated samples on the basis of the slope of the antibacterial assay. The mean slope of seven samples, rejected because of chlorhexidine contamination, was 6.8 $\pm$ 1.39 reflecting the variability in the extent of contamination. The slopes ranged from 5.4 to 8.5 mm/unit. Any samples with detectable chlorhexidine concentrations were discarded.

3.4 Discussion

The antibacterial activity of amniotic fluid has been widely reported, as documented in Chapter 1. However, comparison between these findings and those of other workers may be inappropriate: B. subtilis, a Gram-positive micro-organism is sensitive to antibacterial agents (such as B-lysin) which are inactive against Gram-negative organisms such as E. coli. Most studies merely indicate that a particular bacterial species was susceptible to a certain proportion of tested amniotic fluids. Ford et al. (1981) extended such work in an attempt to quantify, approximately, the extent of bacterial growth inhibitory activity in amniotic fluid using the tube titration technique. As demonstrated by preliminary experiments, and supported by Reeves & Bywater (1975) and by de Louvois (1982), assessment of antibacterial activity by the tube titration technique was inferior to the plate diffusion assay, both in terms of accuracy and reproducability. This is, perhaps, not surprising, as it is impossible to attain consistantly, an accuracy of $\pm$ 25% with a method which provides answers as discontinuous variables in steps of not less than 50%.

The plate diffusion assay revealed antibacterial activity in all amniotic fluids tested. In addition, it could be seen that some of these samples were strongly active such that the activities of the group, as a whole, were logarithmically distributed. Use of this technique also produced a datum not generated by other assay techniques, the slope of the bacterial growth inhibition curve. This provided not only information on depreciation of activity with dilution but also an additional check on contamination of the amniotic fluid sample with exogenous antibacterial components.

The antibacterial activity of amniotic fluid bore no relation

to maturity, parity, maternal age, indication for induction of labour, or source of amniotic fluid. It was, however, strongly influenced by various contaminants. Several groups have reported that the inhibitory effects of amniotic fluid are antagonised by the presence of meconium, and this was confirmed in this study.

In all reported studies of antibacterial activity in amniotic fluid, any sample contaminated with blood was discarded peremptorily because of possible introduction of antibacterial factors from blood into amniotic fluid. This possibility has, heretofore, never been investigated as assays could not distinguish between the factors. The approach is however vindicated by this study. In heavily stained samples, the contamination was such that the contaminating factors exerted a more powerful antibacterial effect than that exerted by amniotic fluid factors.

'Hibitane' cream is used routinely for antiseptic lubrication of the vagina when performing a manual examination for determination of cervical dilation, and when passing a catheter to rupture the fetal membranes. Amniotic fluid collected following this manoeuvre may thus be contaminated with chlorhexidine, especially those samples collected draining <u>per vaginam</u>. Contaminated samples were detected, both by the physiocochemical technique and by analysis of the growth inhibition curve, and discarded.

The slope of the growth inhibition curve produced by amniotic fluid seemed to be somewhat variable. This may have been due to differences in the concentrations of the particular antibacterial amniotic fluid factors, coupled with inter- and intra-assay variations. However, as shown above, it was possible to detect contamination of amniotic fluid with blood or 'Hibitane' by comparison of slopes, from the assay of such samples, with those from uncontaminated samples.

CHAPTER FOUR

The antibacterial activity of high and low molecular weight fractions of human amniotic fluid

As demonstrated in chapter 3, antibacterial activity of amniotic fluid reported in previous studies, was also observed in this study. However, the activity exerted by the low molecular weight component reported by Galask's group (Schlievert et al. 1976a,b,c) and by Sachs and Stern (1979) was the principal focus of attention in this investigation. Therefore, the work reported in this chapter was performed to examine the relative magnitudes of the antibacterial activities of the high molecular weight and low molecular weight fractions of amniotic fluid. Further, the antibacterial activity of the low molecular weight component through the purification sequence was examined.

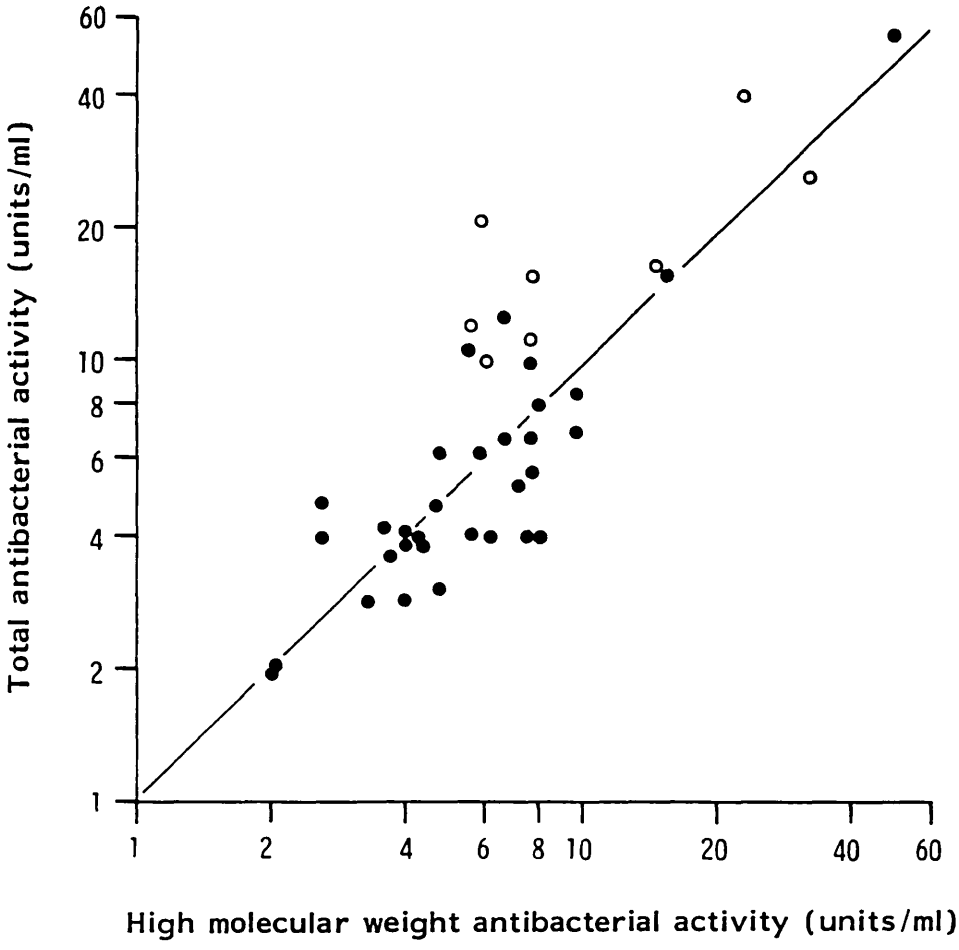


Fig. 7. Correlation of total and high molecular weight antibacterial activities in the presence (o) and absence (●) of low molecular weight antibacterial activity.

The equation for the regression line was,

Total activity = 0.083 + 0.945 (HMW activity)

$r = 0.83$

4.1 Antibacterial activity of fractionated amniotic fluid

In 44 samples of amniotic fluid, the high molecular weight components were retained after separation from low molecular weight components by ultrafiltration (section 2.5.1). The retained material was reconstituted in phosphate-free and zinc-free deionised water. Antibacterial activity was assessed in the whole amniotic fluids, the high molecular weight fractions, and the low molecular weight fractions.

4.1.1 High molecular weight antibacterial activity

Antibacterial activity was present in all samples of whole amniotic fluid (section 3.1.2). The geometric mean of the antibacterial activity in these samples was 7.6 units/ml, with a standard deviation range of 3.6 to 15.9 units/ml. Similarly, high molecular weight antibacterial activity was present in all of the term amniotic fluid specimens tested. The geometric mean activity of these amniotic fluid fractions was 6.5 units/ml with a standard deviation range of 3.4 to 12.4 units/ml. The antibacterial activity in the high molecular weight fraction represented, on average, 93% of the antibacterial activity of the whole amniotic fluid (Fig. 7).

4.1.2 Low molecular weight antibacterial activity

In contrast to both whole amniotic fluid and the high molecular weight fraction, antibacterial activity was demonstrated in the ultrafiltrates of only 9 of the 44 specimens tested. Similarly, of the 122 samples of amniotic fluid described in section 3.1.1, 115 ultrafiltrates were examined but only 29 possessed low molecular weight antibacterial activity. The distinction of the component with low molecular weight antibacterial activity from that with high molecular weight activity was supported by the difference in the slope of the bacterial growth inhibition curves. The gradient of the

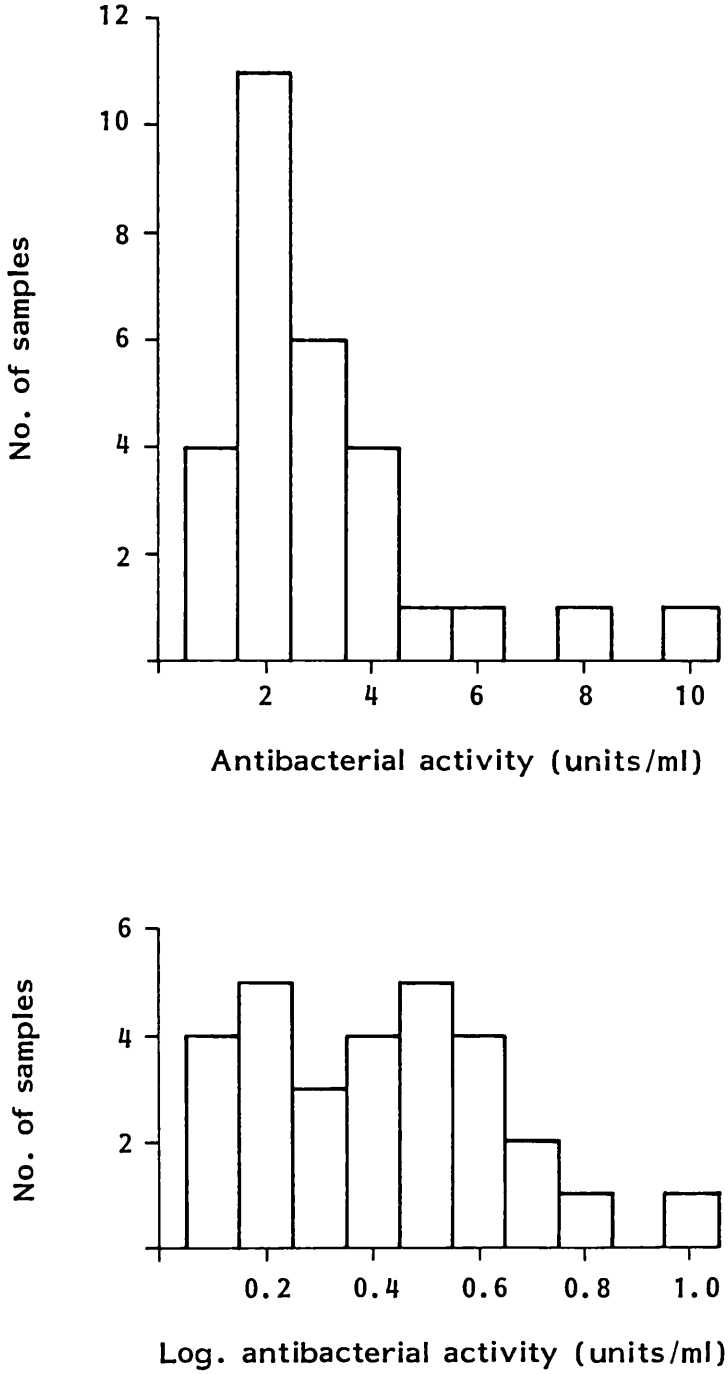


Fig. 8. The distribution of antibacterial activities in 29 amniotic fluid ultrafiltrates, displayed in both arithmetic and geometric forms.

Table 9. Antibacterial activity (units/ml) of amniotic fluid with and without low molecular weight (LMW) antibacterial activity, geometric means (1 s.e. range).

	Ultrafiltrate antibacterial (n = 8)	Ultrafiltrate inactive (n = 32)
Total amniotic fluid activity	19.4 (15.5 - 24.2)	5.6 (5.1 - 6.1)
High molecular weight (B-lysin) activity	13.5 (10.0 - 18.1)	5.5 (5.1 - 5.9)

Total activity was greater ($P < 0.05$, paired t-test) than was accounted for by B-lysin activity when LMW activity was detected.

low molecular weight fraction was 7.5 ± 0.5 mm/unit as compared with 4.9 ± 0.6 mm/unit observed for antibacterial activity in whole amniotic fluid.

As seen with whole amniotic fluid, values for the low molecular weight antibacterial activities also had a positively-skewed, non-normal distribution (Fig. 8). Logarithmic transformation of the data rendered a better approximation to a normal distribution ($P < 0.01$).

4.1.3 Relation between high and low molecular weight antibacterial activities

It has been shown (Fig. 7) that the antibacterial activity of the whole amniotic fluids was directly related to the concentration of high molecular weight antibacterial factor(s), which accounted for most of the activity. In addition, there was a relation between high molecular weight and low molecular weight activity (Table 9): Those fluids with elevated high molecular weight activity also possessed low molecular weight activity. The antibacterial activity of the two fractions seemed to be additive. In fluids with low molecular weight activity, the total antibacterial activity was greater than could be accounted for by their high molecular weight activity (paired t-test, $P < 0.05$), but this was not so when no low molecular weight activity was detected in the ultrafiltrates. It was not demonstrated that the high molecular weight material was identical with β-lysin, e.g. by the use of specific antibodies as described by Ford et al.(1977). However, it is reasonable to assume, on the basis of the experiments in Chapter 5, that these materials would be the same or very similar.

4.2 Antibacterial activity in low molecular weight fractions

4.2.1 Antibacterial activity of lyophilised amniotic fluid ultrafiltrates.

As previously noted, antibacterial activity was demonstrated in the ultrafiltrates of only 9 of 44 amniotic fluids. It was, therefore, of interest to determine whether the low molecular weight substance was absent from the remaining 35 samples or present in concentrations below the limit of detection (1.2 units/ml).

Accordingly, the samples were concentrated 10-fold by lyophilisation and redissolving in zinc- and phosphate-free deionised water, and tested for antibacterial activity to *B. subtilis*. The ultrafiltrates with antibacterial activity all retained activity through this procedure. Seventeen of the inactive ultrafiltrates were concentrated, and 11 of them were found to possess antibacterial activity when concentrated 10-fold. When concentrated 20-fold, activity was detected in all ultrafiltrates. This indicates that the low molecular weight antibacterial component was present in sub-threshold concentrations in these samples of amniotic fluid.

The antibacterial activity, in 17 samples of ultrafiltrates of amniotic fluid with low molecular weight antibacterial activity on initial testing, was examined before and after lyophilisation. Recovery of antibacterial activity after lyophilisation varied between 10% and 65% of initial activity: mean recovered activity was 35% $\pm$ 17%.

4.2.2 Preparation of a crude extract with low molecular weight antibacterial activity

A variety of extraction techniques have been employed for the extraction of peptides from physiological material, as described earlier (section 2.5.3). Extraction of the peptide(s) of interest

Table 10. Extraction of antibacterial activity from lyophilised ultrafiltrates of amniotic fluid.

Sample	Control samples			Extracted samples		
	Activity (units/dl)	Weight (g/dl)	Specific activity (units/g)	Activity (units/dl)	Weight (g/dl)	Specific activity (units/g)
1	42.0	0.937	44.8	28.5	0.492	57.9
2	26.0	0.989	26.3	17.0	0.519	32.8
3	78.5	0.974	80.6	48.5	0.512	94.7
m ± 1 s.d.	48.8 ± 26.9	0.967 ± 0.027	50.6 ± 27.6	31.3 ± 15.9	0.508 ± 0.014	61.8 ± 31.1

$$\text{mean recovery} = \frac{31.3}{48.8} \times 100\% = 64\%$$

$$\text{mean purification} = \frac{61.8}{50.6} \times 100\% = 1.2 \text{ fold}$$

The technique of Byfield et al. (1976) was used to determine whether the low molecular weight antibacterial component could be extracted from lyophilates of amniotic fluid ultrafiltrates.

into organic solutions is employed to effect concentration and/or purification. This approach was investigated for extraction of low molecular weight activity from lyophilates of amniotic fluid ultrafiltrates.

Three samples of 200 mls of amniotic fluid ultrafiltrates, each divided into 4 x 50 ml aliquots, were shell-frozen and lyophilised. Duplicate control lyophilates of each ultrafiltrate sample were simply dissolved in 5 ml assay buffer (ammonium acetate 50 mmol/l, pH 7.4). The second pair of lyophilates were extracted by the method of Byfield et al. (1976). This technique employs 3 extractions with n-butanol:acetic acid:water (100:10:28) using 10 ml organic solution per gram of material to be extracted. The extracted material was pooled and evaported to dryness under a jet of nitrogen gas. The dried material was dissolved in 5 ml assay buffer and tested, together with the controls, for antibacterial activity to B.subtilis. The results are shown in Table 10.

Acid butanol extraction recovered only 64% of the antibacterial activity in the control samples, but with 53% of the weight resulting in only a modest 1.2-fold increase in specific activity. Having confirmed that the antibacterial activity in lyophilates of amniotic fluid ultrafiltrates could be extracted into acid butanol, the technique was modified to optimise the extraction of the antibacterial component.

For the second extraction medium, the water content was reduced and the volatility increased by the substitution of methanol for n-butanol. The composition of the revised medium became methanol:acetic acid:water (100:10:10). As before, duplicate lyophilates, of 50 ml ultrafiltrate, were either dissolved in assay buffer or extracted and dissolved in assay buffer. The antibacterial

Table 11. Extraction of antibacterial activity from lyophilised ultrafiltrates of amniotic fluid.

Sample	Control samples			Extracted samples		
	Activity (units/dl)	Weight (g/dl)	Specific activity (units/g)	Activity (units/dl)	Weight (g/dl)	Specific activity (units/g)
1	89.5	0.982	91.1	65.0	0.421	154
2	78.5	0.984	79.8	53.5	0.415	129
3	43.0	0.956	45.0	31.0	0.397	78.1
m $\pm$ 1 s.d.	70.3 $\pm$ 24.3	0.974 $\pm$ 0.016	72.0 $\pm$ 24.1	49.8 $\pm$ 17.3	0.411 $\pm$ 0.012	120 $\pm$ 39

$$\text{mean recovery} = \frac{49.8}{70.3} \times 100\% = 71\%$$

$$\text{mean purification} = \frac{120}{72.0} = 1.7 \text{ fold}$$

The ability of a modified medium to remove the low molecular weight antibacterial component from lyophilised ultrafiltrates was investigated. The lyophilates were extracted three times with the following medium, methanol:acetic acid:water (100:10:10).

Table 12. Extraction of antibacterial activity from lyophilised ultrafiltrates of amniotic fluid.

Sample	Control samples			Extracted samples		
	Activity (units/dl)	Weight (g/dl)	Specific activity (units/g)	Activity (units/dl)	Weight (g/dl)	Specific activity (units/g)
1	36.5	0.964	37.9	28.5	0.432	66.0
2	49.5	1.011	49.0	40.5	0.448	90.4
3	28.5	0.978	29.1	22.0	0.422	52.1
m ± 1 s.d.	38.2 ± 10.6	0.984 ± 0.024	38.7 ± 9.9	30.3 ± 9.4	0.434 ± 0.013	69.5 ± 19.4

$$\text{mean recovery} = \frac{30.3}{38.2} \times 100\%$$

$$= 79\%$$

$$\text{mean purification} = \frac{69.5}{38.7}$$

$$= 1.8 \text{ fold}$$

The recovery of antibacterial activity into the extraction medium, from the lyophilised ultrafiltrates, was improved by inclusion of sodium chloride (1% w/v) in the extraction medium ($P < 0.02$).

Table 13. Extraction of antibacterial activity from lyophilised ultrafiltrates of amniotic fluid.

Sample	Control sample Activity (units/dl)	Antibacterial activity (units/dl) in acid-alcohol extracts					
		Extraction 1	Extraction 2	Extraction 3	Extraction 4	Extraction 5	Total
1	36.5	17.9 (49%)	11.2 (31%)	2.6 (7%)	1.2 (3%)	-	32.9 (90%)
2	49.5	21.7 (44%)	16.5 (33%)	5.1 (10%)	1.2 (2%)	1.3 (3%)	45.8 (93%)
3	28.5	12.0 (42%)	10.3 (36%)	2.6 (9%)	1.4 (5%)	-	26.3 (92%)
m ± 1 s.d.	38.2 ± 10.6	17.2 ± 4.9	12.7 ± 3.4	3.4 ± 1.4	1.3 ± 0.1		35.0 ± 9.9
	100%	45%	33%	9%	3%	(1%)	92%

The extraction of antibacterial activity from lyophilised ultrafiltrates by successive volumes of extraction medium was investigated.

activity in each sample was then assessed in the plate diffusion assay. The revised extraction technique resulted in marginal improvements in both recovery of antibacterial activity and specific activity, with 71% of the activity being recovered with 42% of the weight. The results appear in Table 11.

Both Gomes (1955) and Girgis et al. (1985) employed sodium chloride in their extraction media to improve recovery of polypeptides (Girgis 1984, personal communication). The composition of the extraction medium was revised to include sodium chloride (1% w/v), and used as described previously. The recovery of antibacterial activity was then assessed in the agar plate diffusion assay. As demonstrated in Table 12, inclusion of sodium chloride in the extraction medium resulted in improved recovery of antibacterial activity ($P < 0.02$) to 79%. However, the weight recovered in the extracted fraction was equivalent to 44% of that of the lyophilate producing a net purification factor of 1.8-fold.

The purification procedure was examined to maximise the extraction of antibacterial activity. The scheme uses 3 x 10 ml extraction medium per gram of lyophilate. The recovery of antibacterial activity in each 10 ml extraction volume was studied to determine the optimum number of extractions. Accordingly, 3 samples of duplicate 200 ml lyophilised ultrafiltrates were extracted 5 times with medium. Each 10 ml extract was dried down individually, and assessed for antibacterial activity. The results in Table 13 are presented both in terms of absolute antibacterial activity and are also expressed as a percentage of that recovered from control lyophilates.

From the data above, the arbitary use of 3 volumes of extraction medium was vindicated. Approximately 87% of antibacterial

Table 14. Acid alcohol extraction of antibacterial activity from 30 samples of lyophilised ultrafiltrates of amniotic fluid.

Lyophilised ultrafiltrates			Acid alcohol extracts			
Activity units/dl	Weight g/dl	Specific activity units/g	Activity units/dl	Weight g/dl	Specific activity units/g	Recovery %
68.5	1.0300	66.5	51.2	0.2595	197	74.7
102.3	0.9900	103.3	94.0	0.2830	332	91.9
76.2	0.9920	76.0	48.6	0.1900	226	63.7
35.3	0.9520	37.1	28.4	0.2655	107	80.3
49.6	0.9840	50.4	44.4	0.3305	134	89.5
24.6	0.9770	25.2	21.3	0.3025	70	86.6
40.8	0.9100	44.8	36.4	0.2653	137	89.2
44.8	0.9500	47.2	44.0	0.2765	159	98.2
94.9	0.9880	96.1	90.2	0.3100	291	95.1
441.5	0.9310	474.2	411.0	0.2400	1712	93.1
24.0	1.1290	21.3	21.2	0.3865	55	88.4
27.1	0.9365	29.0	25.3	0.2720	93	93.4
29.0	0.9640	30.1	27.4	0.3110	88	94.7
20.8	1.0285	20.2	20.2	0.2915	69	97.1
61.3	0.9710	63.1	44.5	0.3160	141	72.6
27.9	0.9755	28.7	25.3	0.3250	78	90.7
31.5	0.9760	32.3	29.9	0.2680	112	95.1
35.0	0.9440	37.1	30.6	0.3124	98	87.4
58.4	1.0120	57.7	55.3	0.2864	193	94.7
90.1	1.0040	89.7	93.3	0.3220	290	103.6
18.9	1.0240	18.5	16.6	0.3842	43	87.8
330.0	0.9680	340.9	291.0	0.2507	1160	88.2
47.3	1.0610	44.6	43.3	0.3417	127	91.5
39.8	0.9540	41.7	37.1	0.2773	134	93.2
34.7	0.9640	36.0	32.5	0.2582	126	93.7
23.8	1.0240	23.2	22.2	0.3421	65	93.3
107.5	0.9650	111.4	104.0	0.3090	337	96.7
185.0	1.1150	165.9	192.0	0.3610	532	103.8
16.1	0.9900	16.3	14.5	0.2960	49	90.1
35.0	0.9830	35.6	31.7	0.2890	110	90.6

activity in the lyophilised ultrafiltrate was recovered with the first 3 alcohol extracts. Further extractions merely contributed a further 3% of activity but added more sodium chloride (100mg per extraction) with a concomitant reduction in specific activity (i.e. antibacterial activity per weight of extract).

The lyophilate of 1 dl ultrafiltrate weighed approximately 1 g (0.99 ± 0.05 g, $n = 28$). It was therefore more expedient to add extraction medium on a "volume per volume" (of pre-lyophilised ultrafiltrate) rather than a "volume per weight" basis. The extraction volume thus became 10 ml/dl rather than 10 ml/g, this was reduced to 2 ml/dl to facilitate handling, drying and to minimise salt accumulation.

The protocol following was that finally adopted for preparation of extracts with low molecular weight antibacterial activity. Ultrafiltered amniotic fluid (200 ml) was shell-frozen and lyophilised. The lyophilate was "wetted" with 1 ml absolute methanol, and then extracted three times with 2 ml/dl of extraction medium (methanol:acetic acid:water, 90:10:5; sodium chloride 1% w/v). Insoluble material was removed by centrifugation (2000g, 10 minutes). The extracts were combined, evaportated under nitrogen, dried *in vacuo* and re-dissolved in 2 ml assay buffer. Antibacterial activity was assessed in both lyophilate extracts and controls. The results of 30 such extractions are presented in Table 14.

Values for antibacterial activity detected in both ultrafiltrate lyophilates and acid alcohol extracts, like those of the ultrafiltrates, had positively-skewed, non-normal distributions. Logarithmic transformations were used in statistical calculations, and hence values quoted are geometric means $\pm$ 1 standard deviation. The mean activity of the lyophilised ultrafiltrates was 49.6 units/dl

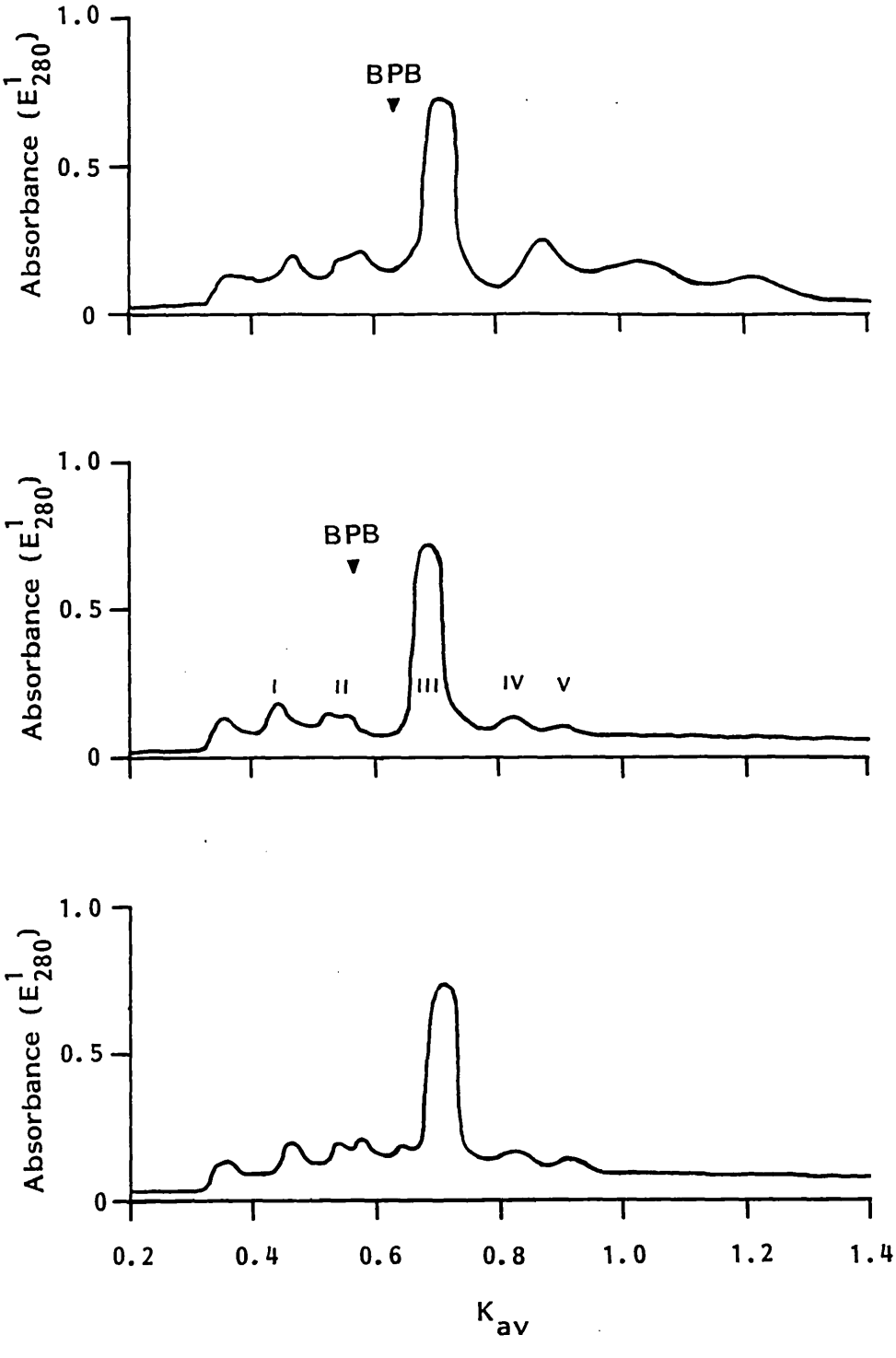


Fig. 9. Determination of the molecular weight of the low molecular weight component of amniotic fluid, with antibacterial activity.

Samples of lyophilised ultrafiltrates of amniotic fluid were separated on Sephadex G10 with either distilled water as eluent (upper trace) or 50 mmol/l ammonium acetate, pH 7.4 (centre trace). Samples were also separated on Sephadex G15 using 50 mmol/l ammonium acetate buffer as eluent (lower trace).
BPB - Bromophenol blue

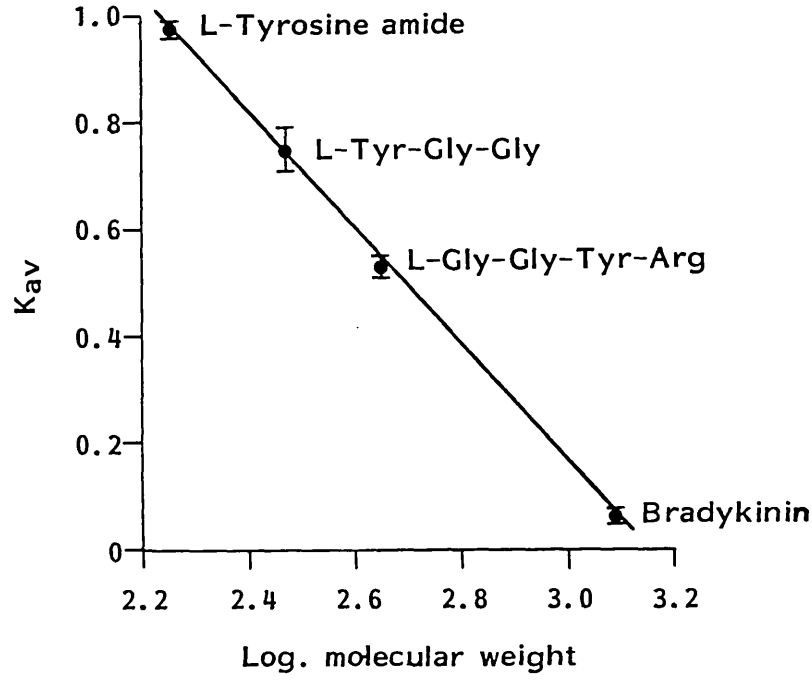


Fig. 10. Molecular weight calibration curve for Sephadex G15.

The calibration curve was constructed using Sephadex G15 (0.9 x 53.3 cm) equilibrated at 4°C with 50 mmol/l ammonium acetate, pH 7.0 at a flow rate of 5.4 ml/hour. One ml aliquots of peptide standards prepared at 1 mg/ ml were used. The elution position (mean ± 1 s.d.) of four aliquots of each peptide standard is indicated.

(22.3 - 111) or 50.2 units/g lyophilate (22.4 - 112). The corresponding activities for the acid alcohol extracts were 44.6 units/dl (19.8 - 100) or 152 units/g extract (64.1 - 359). The overall recovery of antibacterial activity from lyophilate into alcoholic extract was 90.3% with a purification factor of 3.0 $\pm$ 0.34.

Further purification by the method of Byfield et al. (1976), attempted by partition of the extract between formic acid and diethyl ether, was not successful. No activity was recovered from the residues of either phase, neither was any activity detected when these were recombined. Exposure of the acid alcohol extract to formic acid and diethyl ether individually, revealed that the antibacterial activity was resistant to formic acid but inactivated by contact with the organic solvent. Incubation of the extract with ethyl acetate, methyl formate, chloroform or dichloromethane had the same effect.

4.2.3 Determination of molecular weight of the low molecular weight antibacterial component of amniotic fluid

The chromatograph of lyophilised ultrafiltrate separated on Sephadex G10 in distilled water is shown in Fig. 9a, and may be compared with that elution pattern obtained by separation of the lyophilised ultrafiltrate in ammonium acetate on both Sephadex G10 and G15 (Figs. 9b and 9c respectively). The elution position of bromophenol blue is also given for reference. Elution coefficients (K_{av}) were calculated for the eluting UV-peaks. The peak eluting slowest from Sephadex G10 in distilled water had a K_{av} of 1.2.

The column containing Sephadex G15 was calibrated with the peptide standards listed (section 2.5.4). The calibration curve produced is shown in Fig. 10, each point representing the mean of four determinations. Six samples of "crude extract" were

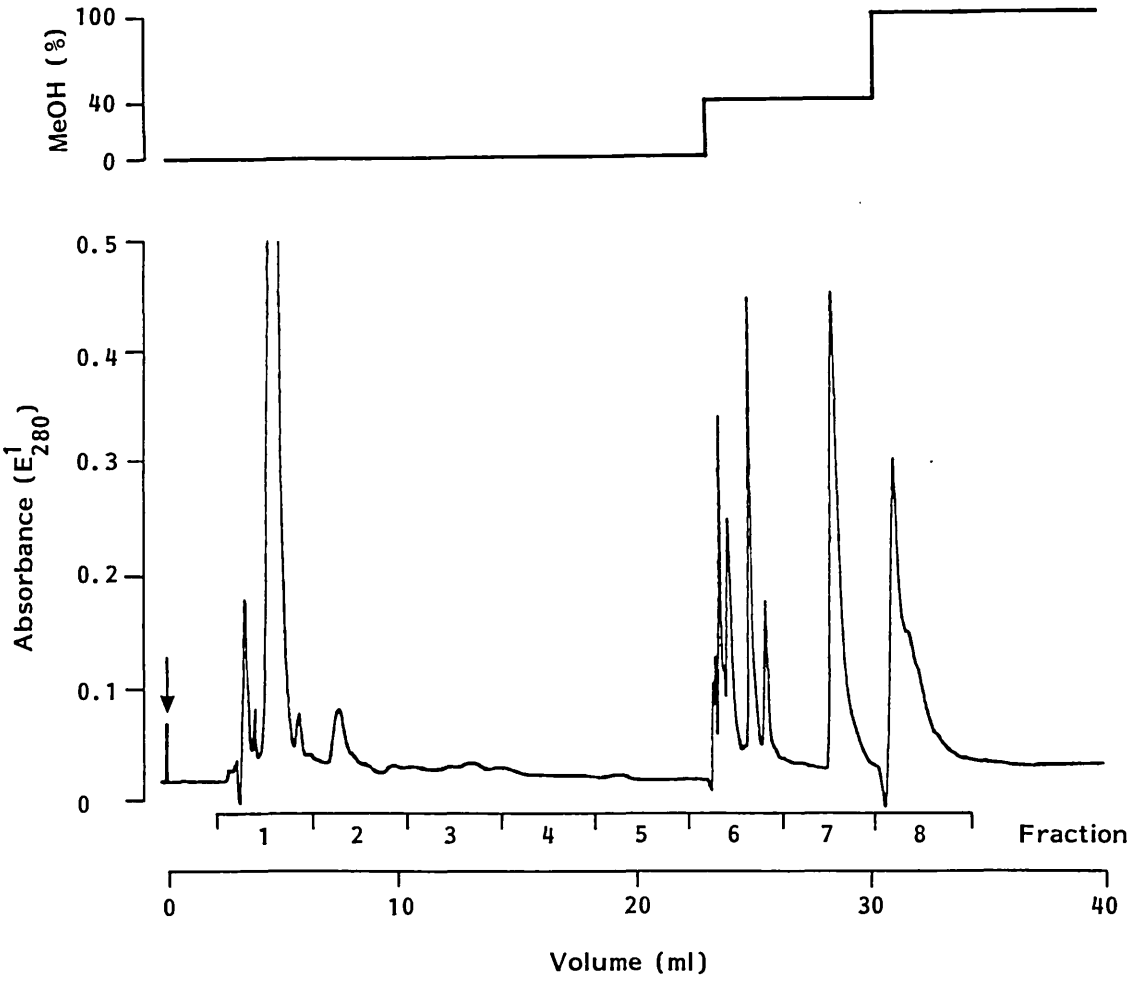


Fig.11. Separation of the components of an acid alcohol extract by reverse phase high performance liquid chromatography. The column (Zorbax ODS 4.6 x 250 mm) was equilibrated at 1.0 ml/min with 50 mmol/l ammonium acetate buffer, pH 7.0 and 20 µl extract injected (arrow) onto the column. Following elution of polar material, apolar components were eluted with 40% and 100% methanol. The eluent was monitored for absorption at 280 nm and collected in 4 ml fractions.

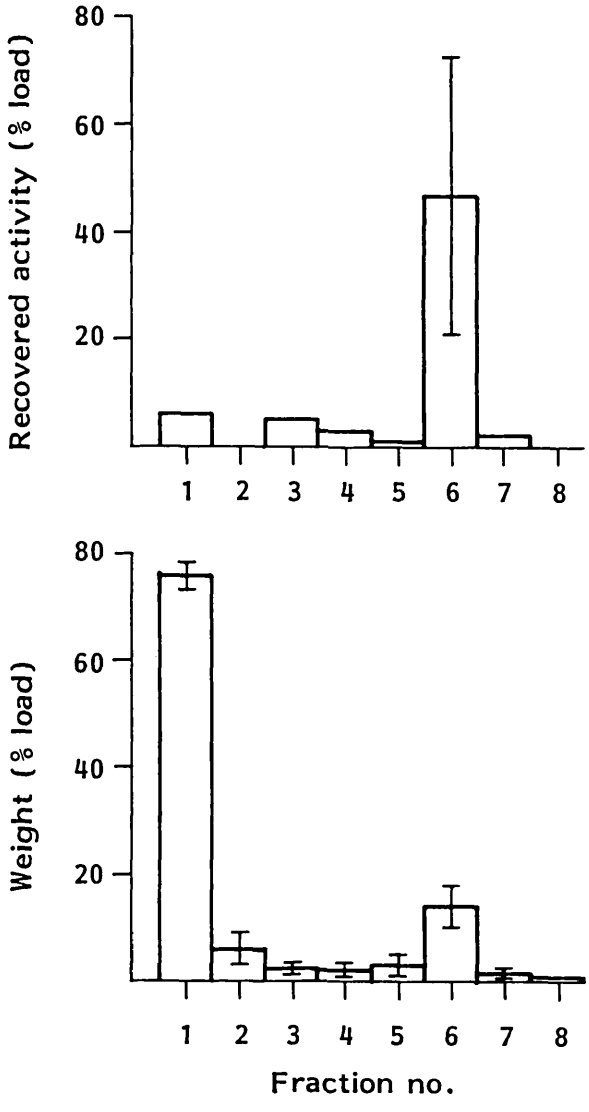


Fig. 12. The distribution of antibacterial activity and weight in HPLC fractions, following separation of acid alcohol extracts by the technique elaborated in the previous figure.

chromatographed and the antibacterial activity of each fraction determined. The fraction containing the maximal activity was used to calculate the elution coefficient. By comparison with the standard curve, the elution coefficient gave an estimated molecular weight of 450 for the low molecular weight antibacterial component.

Recovery of antibacterial activity was 29% ± 4%. In an attempt to increase recovery, the seven fractions around that with maximal activity were pooled, freeze-dried and assayed for antibacterial activity. Further activity had been lost during the lyophilisation procedure, as the recovery of activity had fallen further to 17%± 3%.

4.2.4 Separation of components of the acid alcohol extract by high performance liquid chromatography

A typical chromatograph obtained following reverse phase high performance liquid chromatography (HPLC) of an acid alcohol extract (20 ul) of a lyophilised ultrafiltrate of amniotic fluid is shown in Fig. 11. The extract contained components that ranged from the extremely polar and which eluted at the solvent front , to the apolar components which required methanol elution for recovery from the column. In initial experiments, the eluent was collected in 1.0 ml fractions to determine the elution position of the component with antibacterial activity. Subsequently, the fraction size was increased to 4.0 ml to ensure that the eluting antibacterial activity was collected as one fraction (6).

The distribution of antibacterial activity and weight in eluted fractions is presented graphically in Fig. 12. The mean activity loaded onto the HPLC column was 50.9 (17.4 - 149) antibacterial units. Activity was always recovered in fraction 6, which is therefore depicted with standard deviation bars on the activity distribution graph (Fig. 12). Activity was occasionally

Table 15. The relation of antibacterial activity recovered following reverse phase HPLC to loaded activity.

Loaded Activity (units)	Number of samples	Recovered Activity (% load)
63.8	1	34.9
54.4	1	36.0
44.0	3	30.4
6.0	1	0
65.3	4	36.5
76.4	1	41.1
435.0	2	83.5
401.0	2	91.7
12.3	1	0
94.2	1	43.9
20.2	2	7.9
89.3	1	50.7
46.0	3	23.4
20.2	2	20.0
41.3	4	34.3
24.0	3	6.3
32.4	3	16.4
137.0	3	55.5
29.0	1	16.2
18.1	1	4.7
37.2	4	28.5
263.0	3	79.6

Antibacterial activity eluted from the HPLC column was monitored following injection of 1.0 ml samples of acid alcohol extracts of lyophilates of amniotic fluid ultrafiltrates.

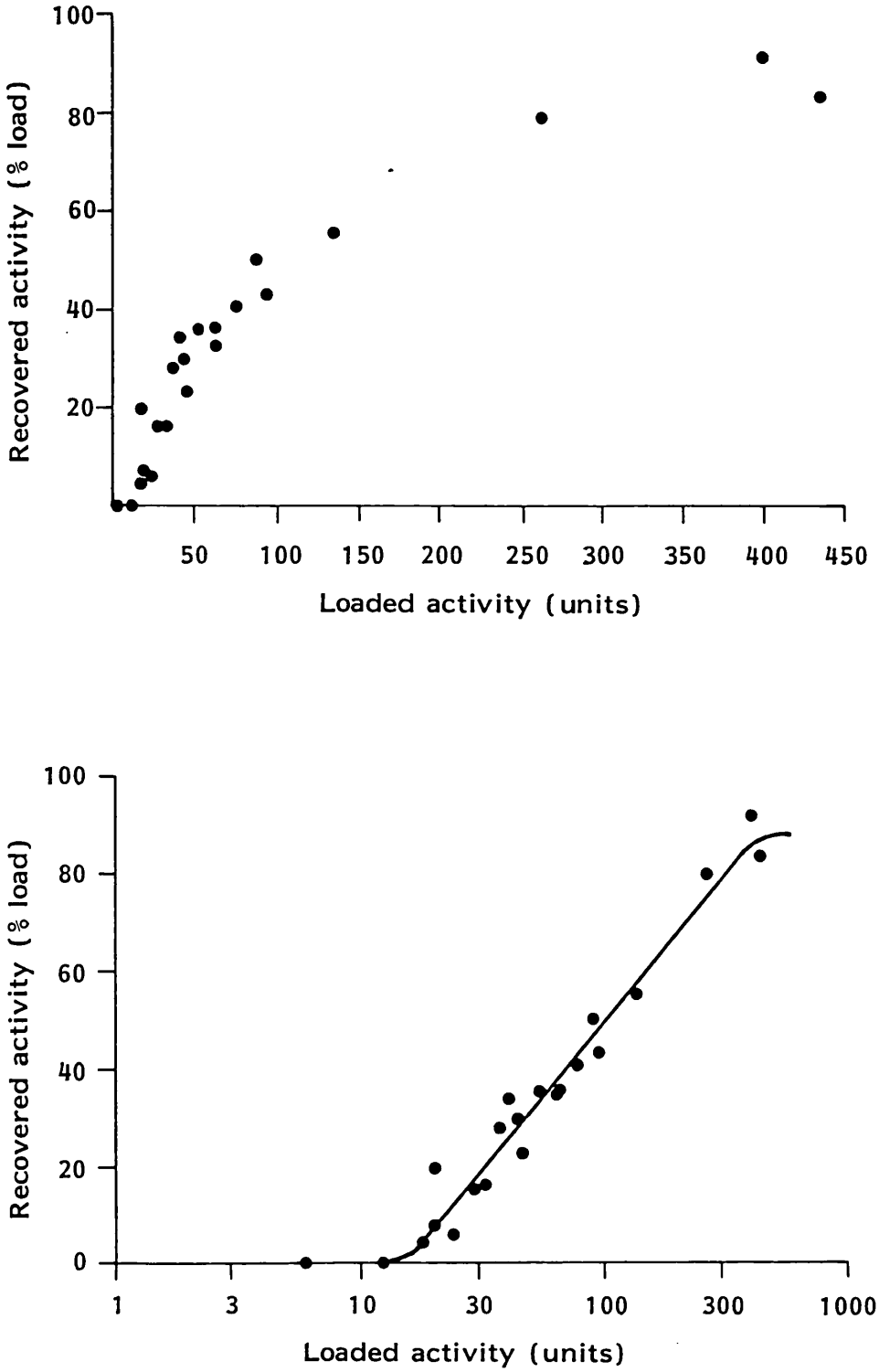


Fig. 13. The relationship of antibacterial activity recovered following HPLC, to the activity of the acid-alcohol extract loaded.

detected in other fractions. In some chromatographs, small amounts of antibacterial activity were detected in the void volume (fraction 1), which was thought to represent column overloading. Activity was only detected in fractions 3 and 4 following chromatography of the two extracts with very high activity (435 and 401 units/ml, respectively).

The mean weight of the acid alcohol extracts loaded onto the column was 272 ± 28.6 mg, of which an average of 118 ± 9.1 mg was accounted for by the presence of sodium and potassium chlorides. Three-quarters of the weight of the injected extract was recovered in the void volume (fraction 1), while 14% was associated with the antibacterial fraction (6).

The recovery of antibacterial activity following separation by reverse phase HPLC of 47 samples of acid alcohol extracts on 22 occasions is shown in Table 15. The relation between recovery of antibacterial activity and the amount of activity loaded onto the HPLC column is shown in Fig. 13. No antibacterial activity was recovered in the eluate if the activity of the sample loaded onto the column, was less than 15 units/ml. Recovery increased in a log. linear fashion with respect to loaded activity, with maximal recovery estimated at 84%.

Following observations that brief exposure of the acid alcohol extract to non-polar organic solvents caused loss of antibacterial activity (section 4.2.2), it was found that long term exposure (16 hours, -20^{o}C) of HPLC fractions to aqueous methanol solutions also resulted in loss of activity.

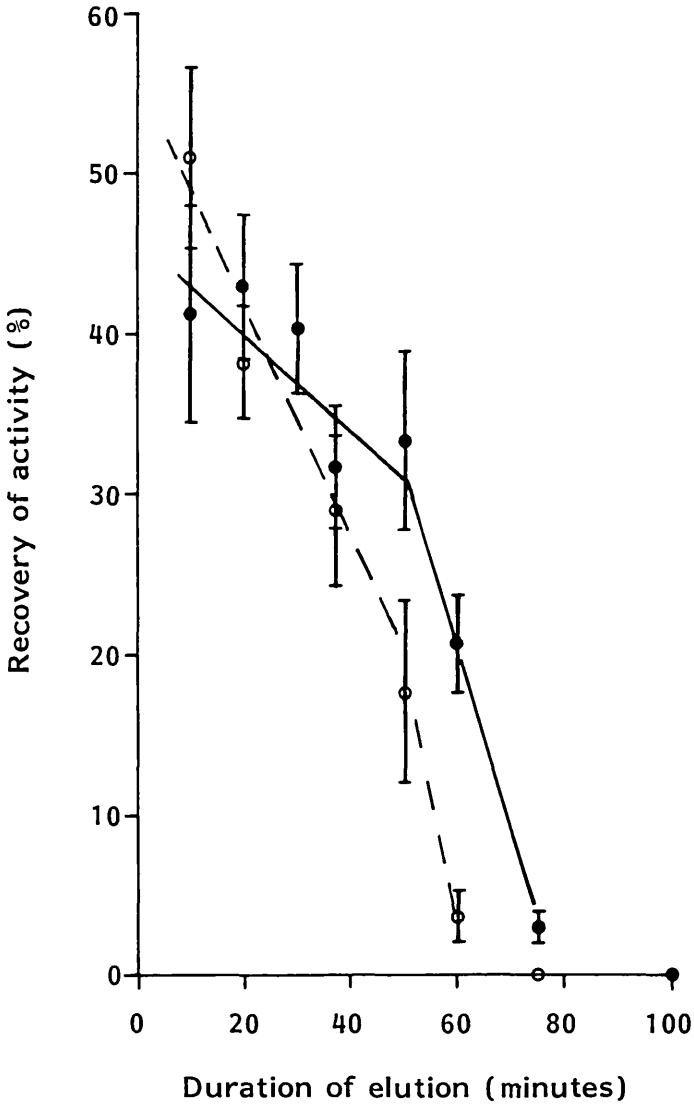


Fig. 14. Duration of elution of the low molecular weight antibacterial component in acid alcohol extracts, from the HPLC system; relation to recovery of activity (mean ± s.d.) under conditions of constant elution volume (30 ml, ●) or constant flow rate (1.0 ml/min, o). Regression lines: continuous - constant volume, interrupted - constant flow rate.

The regression equation, from which the regression line shown in Fig. 13 was derived, is given below.

$$y = -68.8 + 59.3x$$

where x is log. antibacterial activity loaded onto the column and y is the recovery of antibacterial activity expressed as a percentage of loaded activity.

Thus the expected recovery for 30 units loaded onto the column was 19% or 5.7 units. Similarly, the expected recovery for 300 units loaded onto the column was 79% or 237 units, while 50% recovery of loaded activity was expected from antilog.((50 + 68.8)/59.3) = 98.9 antibacterial units.

The purification achieved by this separation procedure varied from 0.6-fold to 9.0-fold and was principally dependent upon the recovery of loaded antibacterial activity. The mean activity loaded onto the column was 50.9 (17.4 - 149) antibacterial units. The expected recovery for an activity loading of this size was 32.7% i.e. 16.6 units. The mean weight of the fraction in which this activity would be expected to elute was 40.1 $\pm$ 7.2 mg. Thus the expected mean specific activity for the HPLC fraction would be 16.6/0.0401 = 414 units/g, while the mean specific activity of the acid alcohol extracts was 152 units/g. Use of echelon elution in conjunction with the HPLC separation step, therefore, would be expected to result in an increase in specific activity of 2.7-fold.

The relation of recovery of antibacterial activity to the duration of elution of the low molecular weight component was also investigated. The recovery of activity over a range of time periods but with a constant elution volume (30 ml) is shown in Fig. 14. It was observed that recovery was dependent on the dwell time of the

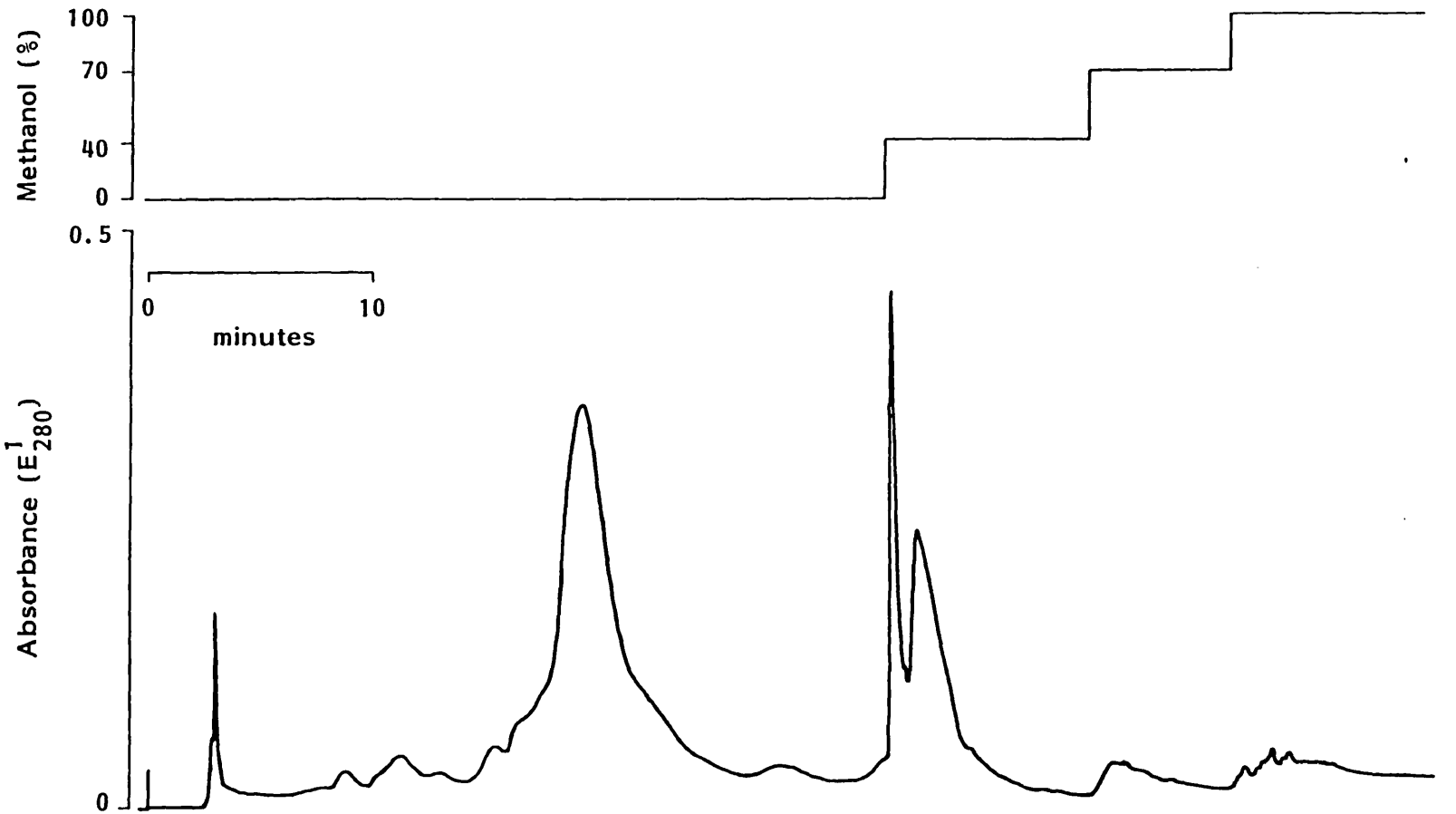


Fig. 15. Refractionation under acid conditions (50 mmol/l acetic acid, pH 3.2) of pooled material with antibacterial activity which had been separated by HPLC at neutral pH.

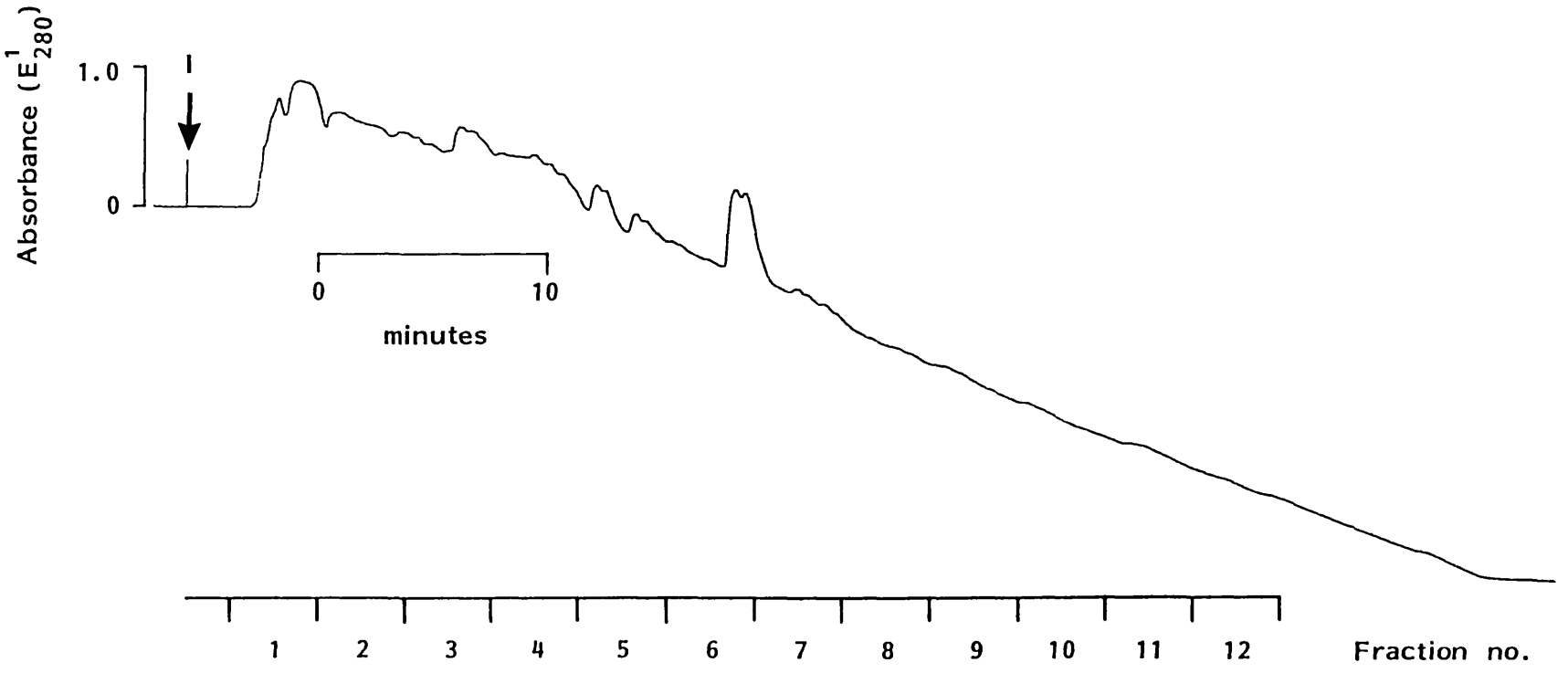


Fig. 16. Separation of the low molecular weight component of an acid alcohol extract of an amniotic fluid ultrafiltrate by gradient elution. The column (zorbax ODS, 4.6 x 250 mm) was equilibrated with 50 mmol/l ammonium acetate, pH 7.0 at a flow rate of 1.0 ml/min. The extract was injected and a methanol gradient simultaneously applied, the concentration changed linearly from 0 to 100% over 50 minutes. The absorbance of the eluent was monitored at 280 nm and collected in 4 ml fractions, which were tested for antibacterial activity to B. subtilis.

Table 16. Comparison of the separational efficiency of HPLC fractionation by echelon and gradient elution.

	Activity (units/ml)	Weight (mg/ml)	Specific activity (units/mg)
Acid alcohol extract	40.9 ± 15.1	281 ± 20.6	0.14 ± 0.05
Echelon elution	12.1 ± 9.4	38.2 ± 3.9	0.33 ± 0.28
Gradient elution	8.1 ± 5.8	5.6 ± 0.8	1.44 ± 1.00

Three samples of acid alcohol extract were assessed for antibacterial activity and loaded onto the HPLC column. The activity and weight of the antibacterial fraction was assessed following both echelon and gradient elution.

antibacterial component on the column. Loss of activity was accentuated when the flow rate was kept constant and the elution volume varied (Fig. 14). Chromatography of the extract was therefore a compromise between purification and recovery of antibacterial activity. The separation employed in Fig. 11 represents the optimised chromatograph for 1 ml of acid alcohol extract.

Further purification was attempted on a number of occasions. Those fractions with antibacterial activity, from several chromatographic separations were pooled, lyophilised and the antibacterial activity assessed. Further purification was then performed by re-chromatography under acid conditions (50 mmol/l acetic acid, pH 3.2). An example of such a separation is illustrated in Fig. 15. A group of contaminants has "moved out" from under the peak fraction in which antibacterial activity was detected.

In contrast to echelon-elution, the low molecular weight antibacterial component was also eluted from the HPLC column by the use of gradient elution, which had become available late in the study. Such a separation is shown in Fig. 16. The relative separational efficiency was assessed by use of three samples of acid alcohol extracts which were fractionated by either echelon or gradient elution. The distribution of mean ($\pm$ 1 s.d.) activities, weights and specific activities of the original extracts are shown in Table 16. By using gradient elution, the fraction containing the activity had a greater purity than that eluted by echelon fractionation (5.6 mg cf. 38.2 mg) but the recovery of antibacterial activity was lower. Nonetheless, use of the gradient elution technique resulted in greater increases in specific activity than that achieved by use of echelon elution ($P < 0.02$).

Table 17. Recovery of antibacterial activity following exposure to ion exchange resins.

Buffer pH											
4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	
39.3	40.8	37.8	33.7	39.3	37.8	32.5	37.8	40.8	39.3	39.3	Activity of buffer controls (units/ml)
											Activity of supernatant (% buffer control)
77		62		63		78		74			CM C-25
72		89		67		59		71			SP C-25
98	98	90	91	95	97	38	0	0	0	0	DEAE A-25

The effect on antibacterial activity, of incubation in buffers of differing pH was assessed. Recovery of activity in the supernatants following exposure of the extracts to ion-exchange resins, was expressed as a percentage of similarly-treated controls.

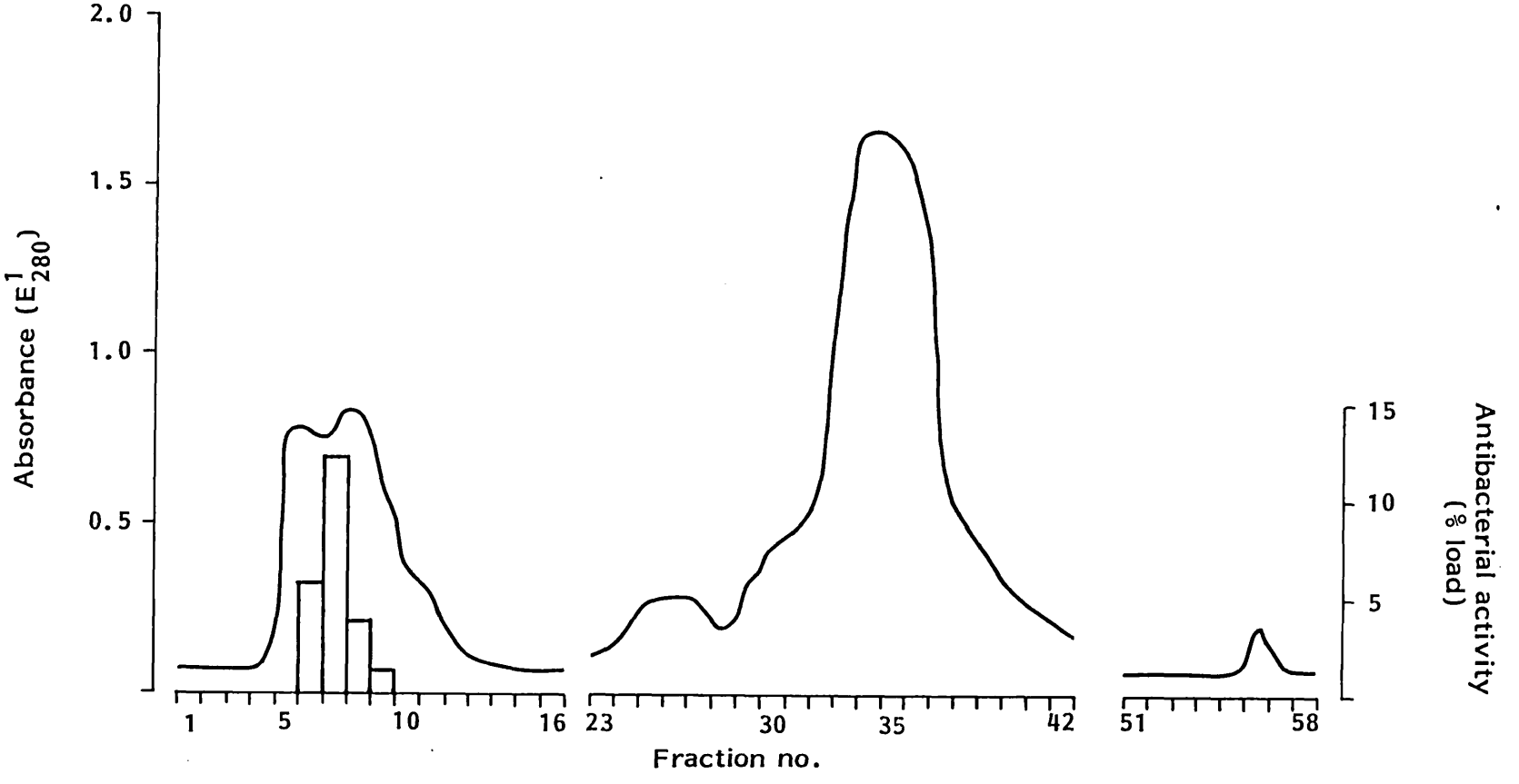


Fig. 17. Separation of the components of an acid alcohol extract, by anion exchange chromatography. The extract was loaded onto a DEAE A-25 column (2.6 x 19.5cm) which had been equilibrated with 10 mmol/l ammonium acetate buffer, pH 7.5. The components of the extract were eluted in a 500ml gradient extending from 10 mmol/l to 1.0 mol/l ammonium acetate buffer, pH 7.5 at 25 ml/hour and collected in 10 ml fractions. A final 50 ml wash of 1.0 mol/l sodium chloride was applied.

4.2.5 Ion exchange chromatography

The test tube method of determination of matrix type, and starting pH, revealed that cation exchange resins were not suitable for separation of the low molecular weight antibacterial substance from other components of the acid alcohol extract. Antibacterial activity was unaffected by the pH of the exchange buffer across the range tested (pH 4 - 9, Table 17). It was present in the supernatants of the weak cation exchange resin, CM C-25, in concentrations equivalent to the controls (Table 17). Antibacterial activity recovered in supernatants after exposure to the strong cation exchange resin, SP C-25, was reduced 30% relative to controls (Table 17). Incubation with zinc acetate (10 µmol/l, 24 hours, 4°C) restored activity.

In contrast, incubation of acid alcohol extracts with the weak anion exchange resin, DEAE A-25, resulted in loss of antibacterial activity in the supernatants when a pH higher than 7.5 was used. A pronounced decrease in antibacterial activity was observed following exposure to DEAE A-25 at pH 7.0. No loss of activity was seen in acid alcohol extracts exposed to DEAE A-25 at a pH lower than 7.0 (Table 17). Incubation of the supernatants of low or no activity with zinc had no effect on antibacterial activity. Since peptides and proteins bind to anion exchange resins at approximately 1 pH unit greater than their isoelectric point (pI), this would suggest that the low molecular weight antibacterial component has a pI near pH 6.5.

Accordingly, 3 ml acid alcohol extracts (39.0 units/ml, 122 units/g) were separated on a DEAE A-25 column as detailed in the methods. The chromatograph is shown in Fig. 17. No activity was recovered in eluted fractions. The activity of these fractions after incubation with 10 µmol/l zinc chloride is also shown in Fig. 17.

Table 18. Extraction of low molecular weight antibacterial activity from amniotic fluid.

	Volume (ml)	Antibacterial activity (units/ml)	Total activity (units)	Weight (g)	Specific activity (units/g)	Recovery (%)
Amniotic fluid ultrafiltrate	100	<1.2 - 11.6 (range)		1.094(±0.052)		100
Lyophilate of amniotic fluid ultrafiltrate	5	9.9 (4.6 - 22.1)	49.6 (22.3 - 111)	0.990(±0.049)	50.2 (22.4 - 112)	42(±9)
Acid - alcohol extraction	1	44.6 (19.8 - 100)	44.6 (19.8 - 100)	0.297(±0.042)	152 (64.1 - 359)	90(±8) 36(±11)
High performance liquid chromatography	1	13.1 (5.4 - 27.7)	13.1 (5.4 - 27.7)	0.040(±0.007)	326 (134 - 691)	37(±25) 14(±13)

Note: Detection limit = 1.2 units/ml. Antibacterial activity expressed as geometric means (s.d.), weight and recovery expressed as arithmetic means (s.d.). For both the acid alcohol extraction and the HPLC purification steps, two values are shown in the recovery column. The upper value is the recovery of activity for that step, and the lower the net recovery of activity.

Recovery of activity was 23% with a concomitant specific activity of 36 units/g. Specific activity of the eluted fractions relative to the loaded sample was 30%. Ion exchange chromatography was therefore not adopted as a purification step.

4.2.6 Summary of purification procedure

The purification scheme finally adopted for the extraction of antibacterial activity from amniotic fluid ultrafiltrates is shown below:

Amniotic fluid ultrafiltrate

↓

Lyophilisation

↓

Acid alcohol extraction

↓

High performance liquid chromatography

The net recovery of antibacterial activity and concomitant purification through this scheme is shown in Table 18. The values quoted for the HPLC step were those which were obtained using the large volume (4 ml) fractions. Those values obtained following collection of 1 ml fractions were similar - the specific activity values were greater but the recoveries smaller. However, neither this collection mode nor the second HPLC step were generally used and hence do not appear in the purification table. Similarly, too few samples were separated by gradient elution on HPLC to make any statistical calculations meaningful. It was not possible to determine the degree of purity of the low molecular weight antibacterial factor in the final HPLC fraction (6).

4.3 Discussion

B-lysin is the main high molecular weight antibacterial component of term amniotic fluid; its identity has been confirmed by Ford et al. (1981) using anti-B-lysin antibody. High molecular weight antibacterial activity was present in all samples of human term amniotic fluids. The low molecular weight component seemed to be of less potential physiological importance. It was detectable in only 29 of 115 samples of amniotic fluid ultrafiltrates, though it was found in sub-threshold levels following concentration of the ultrafiltrates by lyophilisation. The relationship between high B-lysin activity and the presence of the low molecular weight activity suggested that the latter may be associated with the B-lysin molecule. Interestingly, all the early work on the low molecular weight antibacterial factor performed by Galask's group (Schlievert et al. 1976a,b,c, 1977) used ultrafiltration through an Amicon UM10 membrane filter to produce a low molecular weight fraction. Therefore, all tests for "low molecular weight" activity were likely to be contaminated with B-lysin as the molecular weight of this factor is less than 10,000 daltons (section 1.1.3).

The low molecular weight antibacterial component was recovered from lyophilised ultrafiltrates by a procedure used for peptide extraction. The protocol finally adopted recovered the low molecular weight component in good yield (approximately 90%) from the lyophilate. Further purification, to remove lipidic material, caused loss of bioactivity. Recombination of separated lipid material with its extract did not restore activity. Loss of activity was shown to be associated with exposure of the acid alcohol extract to organic solvent. This was a problem also encountered with HPLC separations.

The molecular weight of the antibacterial component was

estimated to be 450 daltons, by size exclusion chromatography. This differs from that previously reported (630, Schlievert et al. 1976c; approximately 670, Sachs & Stern 1979). The fractionation procedure differed significantly from those described in the above studies, both of which used distilled water as eluent. Non-ideal behaviour is observed with this eluent as Sephadex contains a small number of free carboxyl groups (Williams 1972), which cause interaction between charged solutes and the matrix, at low ionic strength. Therefore positively charged molecules are retarded by the matrix, whilst negatively charged substances are excluded from the gel phase and thus elute faster than would be anticipated from molecular size. Values of K_{av} of greater than one, as observed in this study, thus indicated that this phenomenon occured when a buffered eluent was not used. The use of bromophenol blue as a molecular weight marker in such a situation is a confounding problem. It is positively charged in solution and therefore will be eluted later than anticipated from its molecular size. Similarly, the low molecular weight component was thought to be negatively charged (Schlievert et al. 1976c). In distilled water, such a molecule would be eluted ahead of its expected elution position, which would result in an over-estimate of its molecular weight. Use of buffered eluents suppressed these ionic effects, and combined with the use of peptide molecular weight standards was thought to give a more accurate estimate of the molecular weight of the antibacterial component.

The separation of components of the acid alcohol extract by high performance liquid chromatography was subject to large losses of bioactivity. This was thought to be the net effect of several factors - the loss of activity associated with exposure to organic solvents, to the stationary phase and the effects of lyophilisation. However,

this was a technique with a great potential for the purification of the antibacterial component in good yield. Gradient elution would seem to offer the best prospect for further purification work.

The effect of separation on different stationary phases was not investigated as these were not available. Stationary phases vary in their degree of saturation of available silanol groups with C_{18} residues ("carbon loading"). In addition, residual silanol groups may or may not be rendered inactive by reaction with a short chain silane such as ethyl silane or propyl silane, a process known as "end capping". Uncapped silanol groups are highly polar and may bind polar materials irreversibly (Shapiro & Kotthoff 1950). Future work should investigate the use of different C_{18} supports (e.g. µBondapak, Waters Assoc.; Nucleosil C_{18}, Düren; Lichrosorb RP18, Bio-Rad) using methanol gradients of low volume and short duration, for their effects on recovery of the low molecular weight component with antibacterial activity.

The final purification scheme used in this study was not able to produce the low molecular weight antibacterial factor in a form with a high degree of purity. Nevertheless, it was still possible to estimate the approximate molecular weight of this factor and to determine that it carried a slight negative charge in solution at neutral pH.

CHAPTER FIVE

The effects of enviromental factors on the expression of antibacterial activity

As already discussed in Chapter 3, some local enviromental factors (such as blood- and meconium-staining or 'Hibitane' contamination) had a profound effect on the expression of antibacterial activity by amniotic fluid. In addition, it has been shown in Chapter 4, that exposure of the low molecular weight antibacterial component to organic solvents resulted in loss of activity. The extent to which the antibacterial activity is dependent on zinc concentration or inhibited by inorganic phosphorus is controversial (section 1.2.1). This work was undertaken to determine the relation of antibacterial activity to divalent cations, and anions such as phosphate.

Table 19. Amniotic fluid activity (units/ml) in 44 patients and associated levels of total, bound and free, zinc and phosphate.

	Antibacterial activity geometric mean (standard deviation)	Zinc concentration (µmol/l) mean ± s.d.	Inorganic phosphorus concentration (µmol/l) mean ± s.d.
Amniotic fluid	7.6 (3.6 - 15.9)	4.52 ± 2.72	358 ± 155
Reconstituted high molecular weight fraction	6.5 (3.4 - 12.4)	3.09 ± 2.22*	209 ± 115*
Low molecular weight fraction (ultrafiltrate)	0 - 16 (range)	1.43 ± 1.03**	149 ± 90**

* Bound zinc and phosphate levels

** Free zinc and phosphate levels

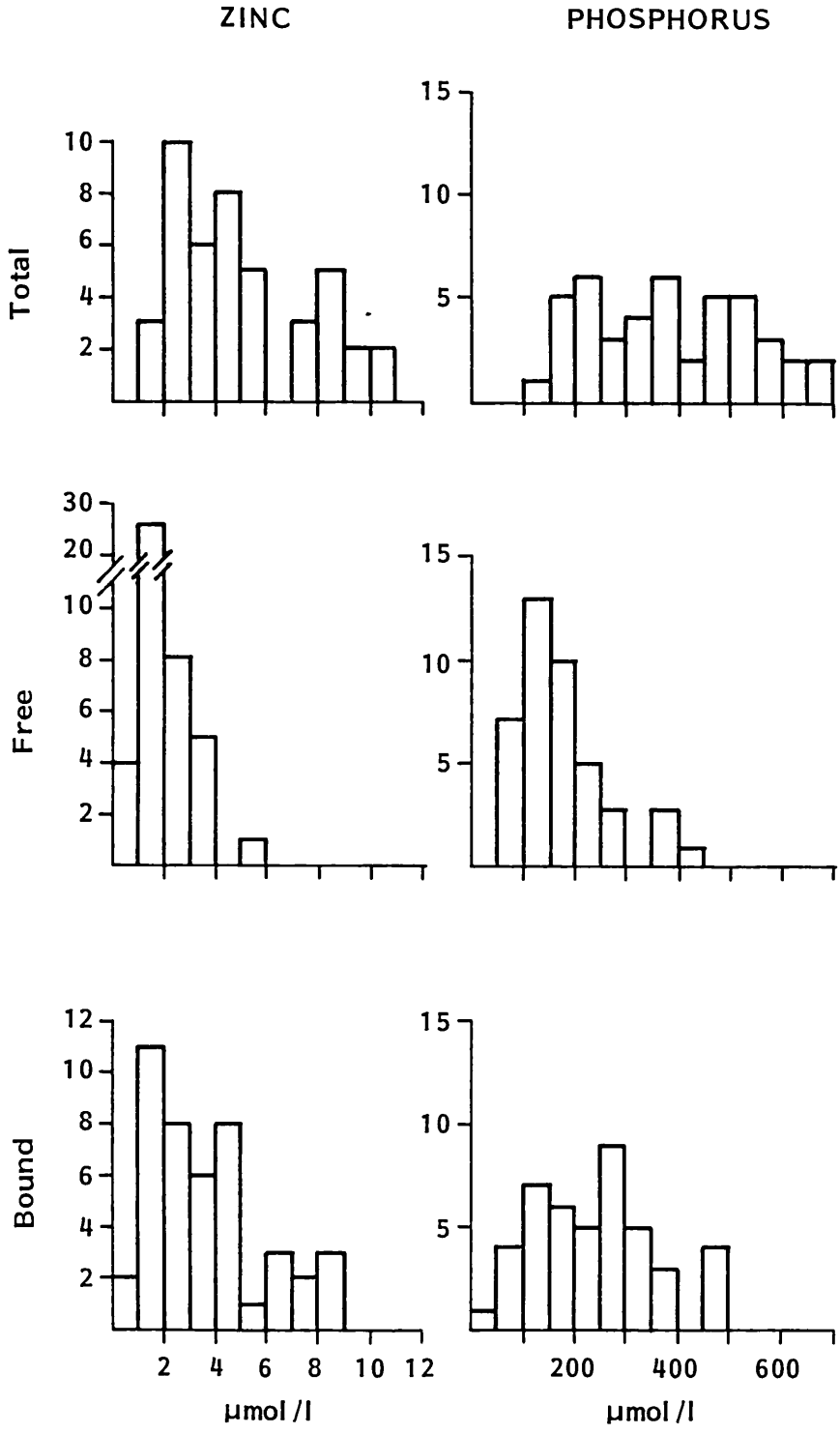


Fig. 18. The distribution of concentrations of zinc and phosphorus in 44 samples of whole amniotic fluid (total values) and in the high (Bound) and low (Free) molecular weight fractions produced by ultrafiltration.

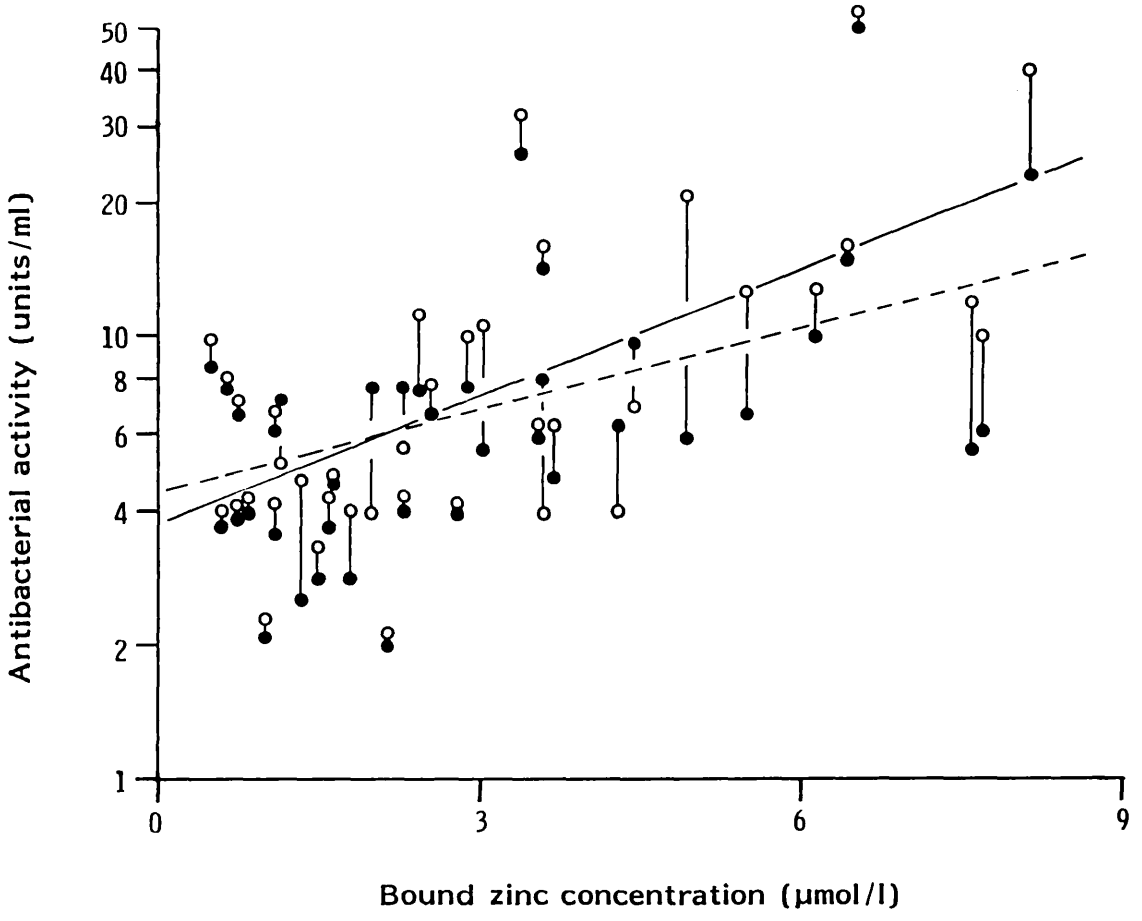


Fig. 19. Total (o) and high molecular weight (●) antibacterial activities (logarithmic scale); relation to bound zinc concentration. Regression lines: continuous-total, interrupted - high molecular weight activities.

5.1 Relation of antibacterial activity to endogenous zinc and phosphorus

Antibacterial activity was present in all samples of intact amniotic fluids and high molecular weight fractions (Table 19), but was present in only a proportion of low molecular weight fractions.

The concentrations of the total, free and protein-bound forms of zinc and phosphorus in amniotic fluid, were determined (Table 19). The distributions of these molecular species in 44 samples of amniotic fluid is shown in Fig. 18. In agreement with Rosick et al. (1983), the concentrations of amniotic fluid zinc exhibited a positively-skewed frequency distribution. Transformation to a logarithmic scale produced a distribution with a better fit to the normal curve. Similarly, free zinc exhibited a positively-skewed frequency distribution, and again a log. transformation rendered a more normal distribution. The geometric mean concentrations for total and free zinc were 3.79 (2.06 - 6.96) and 1.14 (0.59 - 2.22) µmol/l, respectively. Values for bound zinc, together with those of total, free and bound phosphate were approximately normally distributed and log. transformation did not improve the goodness-of-fit to a normal distribution.

Antibacterial activity in whole amniotic fluid was correlated with the concentration of bound zinc ($r = 0.47$, $P < 0.005$; Fig. 19) but no relation to the free zinc concentration was found. The antibacterial activity of whole amniotic fluid was not correlated with the total, bound or free levels of inorganic phosphorus.

The high molecular weight antibacterial activity was measured with the same concentration of zinc and phosphate in the protein-bound forms as was present in the whole amniotic fluid, but in the virtual absence of free zinc (<0.2 µmol/l) and phosphate

Table 20. The antibacterial activity expressed by the high molecular weight (B-lysin) fraction of amniotic fluid in the absence or presence of free zinc (1.5 µmol/l) and phosphate (150 µmol/l).

	Absence	Presence
	30.4	30.4
	5.3	8.7
	3.2	2.4
	14.2	7.0
	4.3	3.8
	30.4	15.2
	5.3	6.3
	5.8	6.4
log mean	0.934	0.877
s.d.	± 0.386	± 0.340
geometric mean	8.58	7.53
	(3.5 - 20.9)	(3.4 - 16.5)

$t = 0.312$ ($0.8 > P > 0.7$)

Table 21. Antibacterial and inactive ultrafiltrates of amniotic fluid; relation to concentrations of zinc and inorganic phosphorus in the original amniotic fluid samples. Mean ± s.e.

	Antibacterial (9 specimens)	Inactive (35 specimens)	Difference
Zinc (µmol/l)			
Free	1.42 ± 0.34	1.43 ± 0.18	-
Bound	5.42 ± 0.78	2.49 ± 0.30	$P < 0.01$
Total	6.85 ± 1.00	3.92 ± 0.39	$P < 0.02$
Inorganic phosphorus (µmol/l)			
Free	154 ± 25.9	147 ± 15.9	-
Bound	243 ± 42.1	201 ± 19.3	-
Total	397 ± 48.0	348 ± 26.7	-

The concentrations of zinc and phosphorus were estimated in 44 samples of whole amniotic fluid (total value) and in the high (Bound) and low (Free) molecular weight fractions produced by ultrafiltration.

(4 µmol/l). The activity was correlated with the concentration of bound zinc ($r = 0.36$, $P < 0.025$; Fig.19) but not to the concentration of free zinc in the original fluid. The activity was not related to the concentration of total, bound or free phosphate in amniotic fluid.

A comparison was made between antibacterial activity in the high molecular weight fraction and that in whole amniotic fluid to determine the consequence of removal of free zinc and phosphate. The correlation coefficient of antibacterial activity was 0.83 ($P < 0.01$; Fig. 7) which indicated that B-lysin activity was independent of the concentrations of free zinc and inorganic phosphorus. In addition, the high molecular weight activity was reassessed in a group of 8 fluids, following addition of a solution which created concentrations of free zinc of 1.5 µmol/l and free phosphorus of 150 µmol/l (cf. free zinc and phosphorus concentrations in Table 19). No difference in antibacterial activity was seen following this manoeuvre (Table 20), which further confirmed that B-lysin activity was independent of the concentrations of free zinc and phosphorus.

Low molecular weight activity had been measured in the absence of bound zinc, but in the presence of the concentration of free zinc present in the whole amniotic fluid specimen. It was of interest to find that this activity was related to the amounts of bound zinc in the original intact amniotic fluid (Table 21), but not to the concentration of free zinc. No relationship was found between low molecular weight activity and either free or bound phosphorus in the whole amniotic fluid (Table 21). To verify the absence of any inhibitory effect of inorganic phosphorus on low molecular weight antibacterial activity, sufficient potassium dihydrogen orthophosphate was added to aliquots of amniotic fluids with

inhibitory ultrafiltrates, as to increase the total phosphorus concentration ten-fold. There was no difference between the activities of the ultrafiltrates before or after the addition of exogenous phosphate.

Table 22. B-lysin antibacterial activity (units/ml; geometric means ± 1-s.d.) of amniotic fluid from 8 patients after incubation and reconstitution, and following exposure to chelating agents.

Chelating agent	Reconstituted amniotic fluid	Chelating agents added before incubation	Chelating agents added after incubation
EDTA		2.7 * (1.3 - 5.3)	8.6 (3.9 - 19.3)
o-PA	9.2* (4.0 - 21.5)	8.8 (3.8 - 20.2)	8.6 (3.6 - 19.0)
Dithizone		8.9 (3.7 - 21.4)	8.8 (3.6 - 21.2)

Whole amniotic fluid was exposed to the chelating agents ethylenediamine tetra-acetic acid (EDTA), ortho - phenanthroline (o-PA), and diphenylthiocarbazone (Dithizone) for the duration of (before) and following (after) incubation at 4°C for 24 hours. The antibacterial activity of the samples was assessed following removal of the chelating agents and compared with similarly treated controls.

* Reduction in activity - $P < 0.02$.

5.2 Dependence of antibacterial activity on divalent cations

The removal of "free zinc" did not affect the expression of antibacterial activity of B-lysin. The effect of the removal of "bound" zinc (and other divalent cations) was investigated.

5.2.1 B-lysin antibacterial activity and the effects of chelating agents

Whole amniotic fluids were all found to exert an antibacterial effect against B. subtilis. Incubation at 4°C for 24 hours and removal of any low molecular weight ("free") antibacterial or other components by ultrafiltration, did not affect antibacterial activity. The activity of whole amniotic fluid was 8.7 (3.7 - 20.4) units/ml while that of amniotic fluid which had been incubated and reconstituted from ultrafiltration residue with PBS was 9.2 (4.0 - 21.5) units/ml.

The main activity of the whole amniotic fluid must be attributed to B-lysin (Ford et al. 1981). Incubation of whole amniotic fluid with EDTA resulted in a mean loss of 79% of this B-lysin antibacterial activity ($P < 0.02$), while incubation with either o-phenanthroline or dithizone had no appreciable effect (Table 22).

5.2.2 Supplementation with divalent cations, of amniotic fluid treated with chelating agents

Prior to supplementation of amniotic fluids with divalent cations, the intrinsic antibacterial effects of these ionic species were assessed. Magnesium and calcium chlorides were non-inhibitory in concentrations up to 1 mol/l. Toxic effects of manganese, iron, cobalt, copper and zinc were observed in concentrations greater than 8.3, 4.5, 1.9, 1.1 and 0.6 mmol/l, respectively.

The first incubation period was that occuring in the presence

Table 23. Antibacterial activity (units/ml; geometric means ± s.d.) of amniotic fluid from 8 patients after incubation and reconstitution, re-incubated in the absence or presence of exogenous divalent cations.

	PBS*	+Mg	+Ca	+Mn	+Fe	+Co	+Cu	+Zn
Added before incubation	9.0 (3.8-21.7)	8.9 (4.4-17.9)	8.6 (3.8-19.7)	9.4 (4.7-19.0)	9.2 (3.9-21.8)	9.6 (4.5-20.6)	9.4 (3.8-18.7)	7.5 (3.4-16.5)
Added after incubation	9.3 (3.6-24.5)	9.7 (4.0-23.7)	9.3 (4.5-19.1)	9.0 (3.6-22.8)	8.5 (3.8-19.0)	8.5 (3.8-18.8)	7.5 (2.4-16.5)	9.7 (4.2-22.5)

* PBS was either added alone or with one of the following divalent cations (added as the chloride): magnesium and calcium, both 5 mmol/l; manganese, iron, cobalt, copper and zinc, all 15 µmol/l.

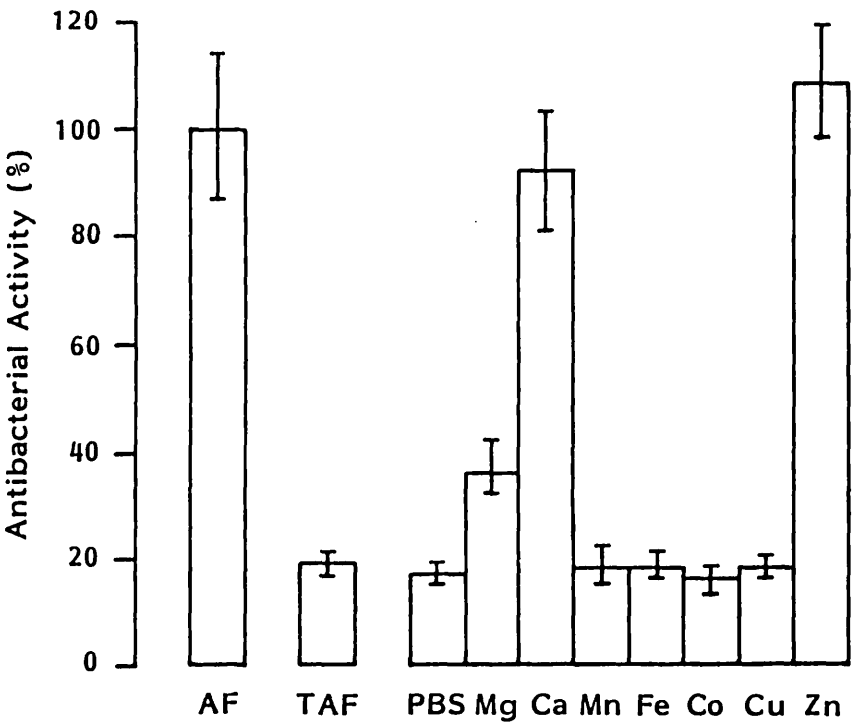


Fig. 20. High molecular weight (B-lysin) antibacterial activity (mean ± 1 s.e.) of 8 amniotic fluids alone (AF), and following treatment with ethylenediamine tetra-acetic acid (TAF). Also shown is the activity of the treated fluids which were subsequently incubated with phosphate-buffered saline (PBS) alone, or PBS which contained magnesium (Mg), calcium (Ca), manganese (Mn), iron (Fe), cobalt (Co), copper(Cu), or zinc (Zn). Acitivity is expressed relative to the untreated fluid.

Table 24. Antibacterial activity (units/ml) of EDTA-treated amniotic fluids following reincubation in the absence or presence of exogenous divalent cations.

	PBS*	+Mg	+Ca	+Mn	+Fe	+Ca	+Cu	+Zn
Added before incubation	2.4 (1.2-5.0)	3.8 (1.9-7.8)	8.3 (3.9-17.6)	2.6 (1.3-5.1)	2.6 (1.3-5.1)	2.2 (1.1-4.4)	2.6 (1.3-5.3)	9.8 (4.2-23.0)
Added after incubation	2.4 (1.2-4.9)	3.0 (1.7-5.5)	2.7 (1.4-5.0)	2.6 (1.4-4.9)	2.3 (1.2-4.5)	2.6 (1.5-4.6)	2.3 (1.1-4.8)	2.5 (1.1-5.7)

* PBS was either added alone or with one of the following divalent cations (added as the chloride): magnesium and calcium, both 5 mmol/l; manganese, iron, cobalt, copper and zinc, all 15 µmol/l.

Table 25. Low molecular weight antibacterial activity (units/ml; geometric means ± 1 s.d.) of amniotic fluid extracts from 8 patients, after incubation at 4°C, and following exposure to the chelating agents ethylenediamine tetra-acetic acid (EDTA), 1, 10-phenanthroline (o-PA), or diphenylthiocarbazone ('Dithizone').

Chelating agent	Reconstituted amniotic fluid	Chelating agents added	
		before incubation	after incubation
EDTA		39.6 (29.1 - 53.9)	38.4 (26.9 - 54.8)
o-PA	38.8* (28.5 - 52.8)	38.7 (26.6 - 56.3)	39.3 (27.0 - 57.2)
Dithizone		3.9* (3.0 - 5.0)	37.1 (26.9 - 51.2)

* Reduction in activity - $P < 0.0001$

or absence of chelating agents, while the second incubation period was that in the presence or absence of divalent cations. The antibacterial activity of the group of fluids which had been simply incubated, ultrafiltered and reconstituted, was unchanged after the second period of incubation (Table 23). The activity was not affected by any divalent cation added either before or after, the second incubation period.

Amniotic fluids whose antibacterial activity had been reduced by treatment with EDTA and reconstituted, were incubated with magnesium, calcium or zinc. This resulted in increases in antibacterial activity (Fig. 20: $P < 0.0002$, $P < 0.0001$, $P < 0.0001$ respectively; paired t-tests). No effect was seen in EDTA-treated fluids supplemented with manganese, iron, cobalt or copper (Table 24). In contrast, simple addition of divalent cations to EDTA-treated fluids after incubation for 24 hours had no effect on antibacterial activity. Supplementation of OPA treated or dithizone treated fluids with divalent cations had no effect on antibacterial activity, either before or after the second incubation.

5.2.3 Low molecular weight antibacterial activity and the effects of chelating agents.

Incubation at 4°C for 24 hours had no apparent affect on low molecular weight antibacterial activity. The mean activity of the extracts was 39.5 (28.1 - 55.4) units/ml before, and 38.8 (28.5 - 52.8) units/ml after incubation. Incubation of the extract with dithizone resulted in a 90% loss of the antibacterial activity, while incubation with EDTA or OPA had no appreciable affect (Table 25).

5.2.4 Supplementation with divalent cations, of amniotic fluid extracts treated with chelating agents.

The antibacterial activity of the extracts which had been only

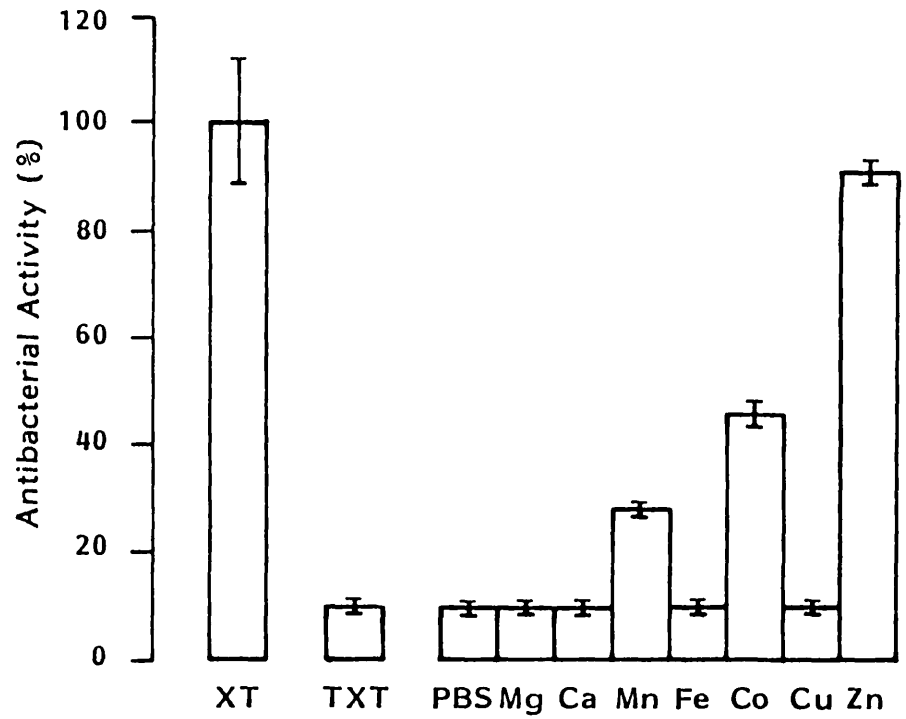


Fig. 21. Low molecular weight antibacterial activity (mean ± 1 s.e.) of 8 amniotic fluid extracts alone (XT), and following treatment with dithiozone (TXT). Also shown is the activity of the treated extracts which were subsequently incubated with phosphate-buffered saline (PBS) alone, or PBS which contained magnesium (Mg), calcium (Ca), manganese (Mn), iron (Fe), cobalt (Co), copper (Cu), or zinc (Zn). Activity is expressed relative to that found in the untreated (amniotic fluid) extract.

Table 26. Antibacterial activity (units/ml) of dithizone-treated acid alcohol extracts following re-incubation in the absence or presence of exogenous divalent cations.

	PBS*	+Mg	+Ca	+Mn	+Fe	+Co	+Cu	+Zn
Added before incubation	3.9 (3.0-5.2)	3.9 (3.0-5.1)	3.9 (2.9-5.1)	10.8 (7.7-15.1)	3.8 (2.8-5.1)	17.8 (12.8-24.7)	4.0 (3.1-5.1)	35.1 (25.8-47.8)
Added after incubation	3.8 (2.8-5.1)	3.8 (2.9-4.9)	4.0 (3.0-5.2)	3.7 (2.7-5.2)	3.9 (2.9-5.2)	3.9 (2.9-5.2)	3.9 (3.0-5.1)	3.9 (3.0-5.1)

* PBS was either added alone or with one of the following divalent cations (added as the chloride): magnesium and calcium, both 5 mmol/l; manganese, iron, cobalt, copper and zinc, all 15 µmol/l.

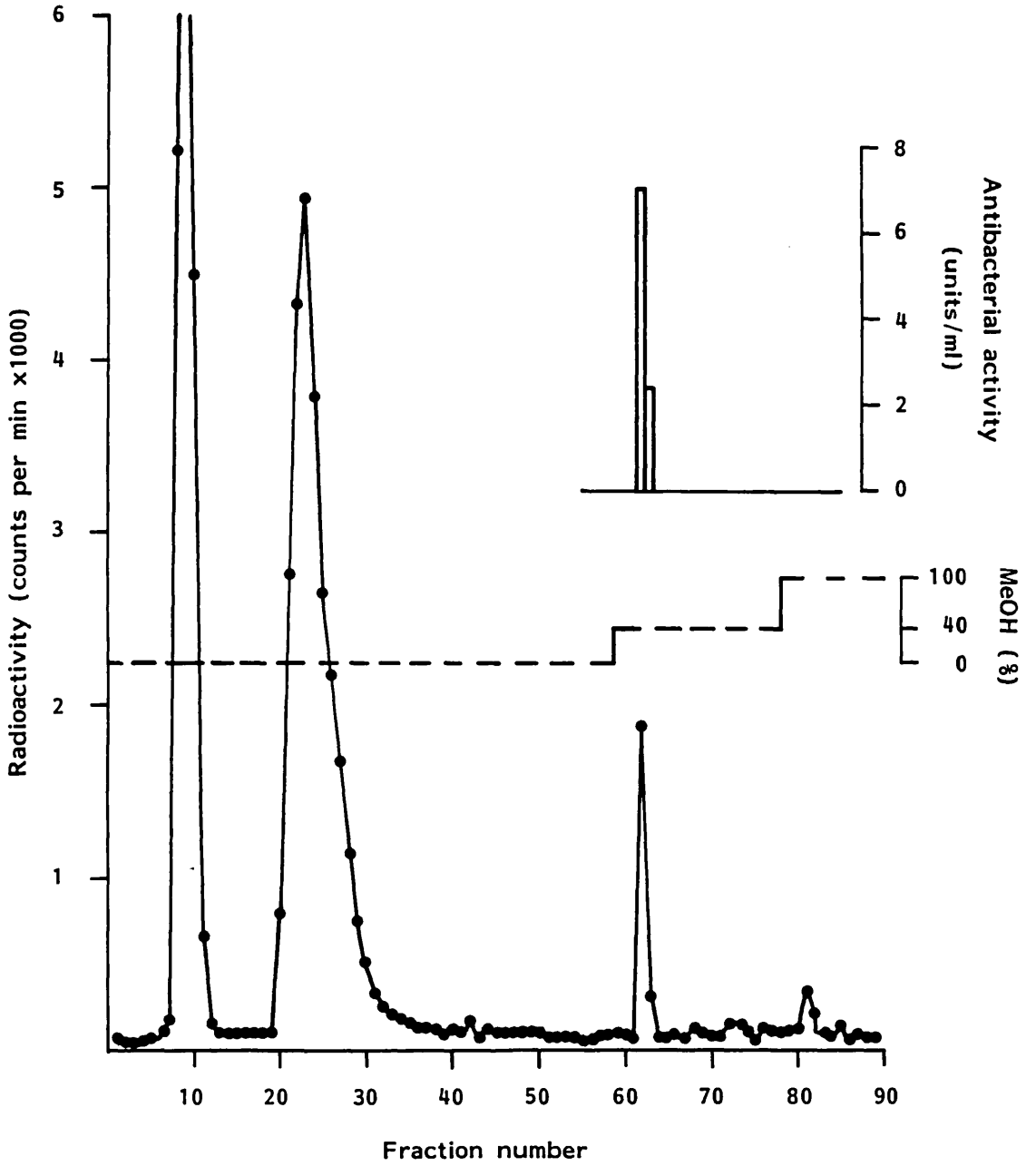


Fig. 22. Fractionation by reverse phase HPLC of dithizone-treated acid alcohol extracts incubated with radio-zinc.

Acid alcohol extracts, of lyophilised ultrafiltrates of amniotic fluid, were stripped of zinc by exposure to the chelating agent, dithizone. Following incubation with radio-zinc and assessment of antibacterial activity, the treated extracts were separated by standard echelon-elution reverse phase HPLC. Recovery of antibacterial activity and radio-zinc in the eluent was studied.

incubated, was unchanged after the second incubation. The activity was not affected by any divalent cation added either before, or after, the second period of incubation.

Extracts, whose antibacterial activity had been reduced by treatment with dithizone, were incubated for 24 hours at 4°C with manganese, cobalt and zinc. This resulted in increases in antibacterial activity (Fig.21; $P < 0.0001$, $P < 0.0001$, $P < 0.0001$) respectively; paired t-tests). No effect was seen in dithizone-treated extracts, supplemented with magnesium, calcium, iron or copper (Table 26). Simple addition of divalent cations to dithizone-treated extracts after incubation had no effect on the diminished antibacterial activity.

The remaining extracts were also incubated for a second time in the absence or presence of one of the divalent cations. Supplementation of EDTA- or OPA-treated fluids with divalent cations had no effect on antibacterial activity, whether added before or after the second incubation period.

5.2.5 Supplementation with radioactive zinc, of acid alcohol extracts treated with dithizone

Three extracts, whose antibacterial activity had been reduced by treatment with dithizone and restored by incubation with radiozinc were fractionated by echelon-elution reverse phase HPLC. Recovery of antibacterial activity and radioactivity following such a separation is shown in Fig. 22. Forty-six percent of radiozinc eluted in the void volume, (fractions 7-12) while 42% eluted in fractions 20 - 37 (Peak A). A very small proportion of radiozinc eluted in fractions 62 - 63 (Peak B). Antibacterial activity eluted principally in fraction 62, although in one case activity was also detected in fraction 63. The ^{65}zinc associated with fraction 62

was present at 120 ± 10 pmol/unit, while that associated with fraction 63 was 45 pmol/unit.

Extracts not treated with dithizone, were also incubated with ^{65}zinc. No radioactivity associated with the antibacterial fraction was collected.

5.3 Discussion

The values for total zinc and phosphorus are similar to those in previous studies (Cantarow et al. 1933; Henkin et al. 1971; Chez et al. 1978; Brandes et al. 1980; Anastasiadis et al. 1981; Gibbs et al. 1982). Schlievert et al. (1976a) whose study included samples of amniotic fluid from pregnancies ranging from 16 to 44 weeks, found comparable zinc levels but higher phosphate concentrations.

The mean level of bound zinc in term amniotic fluid was 3.1 µmol/l equivalent to 74% of total zinc. This is a much lower proportion than that found in serum (92-98%, Prasad & Oberleas 1970) and may reflect the lower protein concentration found in term amniotic fluid, approximately 5% of that in serum (Queenan 1978). The mean bound phosphate concentration was 209 µmol/l, equivalent to 57% of the total phosphate level.

High molecular weight (B-lysin) antibacterial activity, present in all term samples of amniotic fluid, was positively correlated with the concentration of bound zinc in the amniotic fluid. B-lysin-activity was not related to free zinc, free phosphorus or bound phosphorus, neither was the activity related to the ratios of endogenous concentrations of any form of phosphorus with any zinc species.

Human serum B-lysin is dependant upon calcium ions for full expression of antibacterial activity (Jacox 1950, Myrvik et al. 1958). Magnesium, barium and cadmium in millimolar concentrations are possible substitutes for calcium (Carrol & Martinez 1981). Ford et al. (1981b) have shown that the antibacterial activity on E. coli of amniotic fluid B-lysin, was reduced by treatment with the chelating agent o-phenanthroline, using a 4 hour incubation at 37°C. The activity was restored by zinc but not by addition of magnesium,

calcium, manganese or cobalt. The concentrations used (1.5 - 3.0 µmol/l) were sub-physiological for the former cations: Amniotic fluid at term contains calcium in concentrations estimated between 1.5 and 3.0 mmol/l, and magnesium in concentrations between 0.5 and 2.0 mmol/l (Johnell & Nilsson 1971, Goodlin 1972).

Ethylenediamine tetra-acetic acid (EDTA) chelates magnesium and calcium from solution, o-phenanthroline chelates all group IIb cations, while dithizone at neutral pH is relatively specific for zinc (Margerum & Santacana 1960). In these studies, high molecular weight (B-lysin) antibacterial activity was reduced following cation chelation with EDTA, but not with o-phenanthroline or dithizone. It was not only restored by magnesium and calcium in millimolar concentrations, but also by zinc in micromolar concentrations. High molecular weight antibacterial activity in amniotic fluid is therefore biochemically similar to serum B-lysin. Interestingly, serum B-lysin only exhibits 17% of its initial activity in the absence of divalent cations (Carrol & Martinez 1981a). Amniotic fluid B-lysin activity, following chelation of divalent cations, also displayed 19% of its original activity.

Thadepalli et al. (1982) asserted that the principal bacterial growth inhibitor in amniotic fluid was transferrin and that amniotic fluid was inhibitory only if the concentration of iron was less than 1.2 µg/ml (21 µmol/l). The concentration of iron in amniotic fluid at term was approximately 1.0 µg/ml (18 µmol/l). Supplementation of amniotic fluid with iron (15 µmol/l) failed to render the inhibitory fluids, non-inhibitory. This suggested that transferrin was not a major contributor to the antibacterial effect exerted by amniotic fluid.

The low molecular weight activity was related to the

concentration of protein-bound zinc. It was suggested that amniotic fluid contained a protein carrier responsible for supplying zinc to the low molecular weight component (Scane & Hawkins 1984). The study also showed that the antibacterial activity of the low molecular weight component was dependent upon the presence of divalent cations. Activity was lost following treatment with dithizone and not EDTA or o-phenanthroline, which suggested that zinc was the important cation. Since the low molecular weight component, after cation chelation, required exposure to the cation for some time before regaining activity, the cation may have to be incorporated into the molecule. Serum B-lysin also requires incubation with the cation before it becomes active (Carrol & Martinez 1981a).

Peptides containing glutamate or tyrosine are able to bind zinc which may result in a change in conformation (Bere & Helene 1979). It is not surprising that the pharmacological activity of such peptides is affected by the presence of divalent cations. Schild (1969) showed that the weak effects of lysine-vasopressin, arginine-vasopressin and ornithine-vasopressin, in contracting smooth muscle, were greatly potentiated by divalent cations, while highly active polypeptides such as oxytocin were weakly potentiated. It was thus possible that glutamate or tyrosine could be part of the low molecular weight antibacterial component. This proposal is supported by the work of Schlievert et al. (1976c), which suggested that the low molecular weight antibacterial component of amniotic fluid was a peptide containing glutamic acid.

CHAPTER SIX

Characterisation of the low molecular weight antibacterial component of amniotic fluid

The low molecular weight antibacterial factor of amniotic fluid has been shown to be a small apolar component (chapter 4). The activity of which was dependent upon the presence of divalent cations (chapter 5). The work reported in this chapter demonstrates the chemical nature of the low molecular weight component, as determined by enzymology, affinity chromatography and thin layer chromatography. The antibacterial activity of the low molecular weight component against a limited spectrum of bacteria was also investigated. A series of peptides, the composition of which was based on mass spectrographic analysis of the low molecular weight factor, was then synthesised and tested for antibacterial activity.

Table 27. Recovery of antibacterial activity following exposure of acid alcohol extracts to enzyme solution.

Trypsin	Chymotrypsin	Carboxypeptidase	Staphylococcal protease	Amylase	Glycopeptidase
104.9	104.1	5.2	90.1	90.0	97.5
90.0	97.5	ND	94.6	102.8	104.3
101.3	103.2	2.3	87.5	98.4	93.8
98.7 ± 7.8	101.6 ± 3.6	2.5 ± 2.6	90.7 ± 3.6	97.1 ± 6.5	98.5 ± 5.3

Results are expressed as a percentage of non-enzyme treated controls. ND = not detected.

Table 28. Recovery of antibacterial activity following exposure of acid alcohol extracts to lectin affinity matrices.

		Con A-Sepharose			Wheat germ lectin-Sepharose			Soybean lectin-Sepharose		
		1	2	3	1	2	3	1	2	3
wash in		-	-	-	-	-	-	-	-	-
wash off	1.	42.3	57.8	60.6	86.8	78.6	71.1	65.4	53.2	63.1
(unbound)	2.	12.7	-	-	-	-	11.4	-	23.5	-
	3.	-	-	-	-	-	-	-	-	-
	4.	-	-	-	-	-	-	-	-	-
elution	1.	-	-	-	-	-	-	-	-	-
buffer I	2.	-	-	-	-	-	-	-	-	-
	3.	-	-	-	-	-	-	-	-	-
elution	1.	-	-	-	-	-	-	-	-	-
buffer II	2.	-	-	-	-	-	-	-	-	-
	3.	-	-	-	-	-	-	-	-	-
recovery		57.8 ± 2.8%			82.6 ± 4.1%			68.4 ± 7.3%		

6.1 Enzymology

The results of exposure of acid alcohol extract to various enzymes is summarised in Table 27. Exposure to either trypsin or chymotrypsin had no effect on the antibacterial activity of the extract. Similarly, exposure of the extracts to either amylase or glycoprotease had no discernable effect on antibacterial activity. Exposure of the extract to carboxypeptidase for 4 hours caused complete loss of activity, while exposure to staphylococcus protease caused slight but significant loss of activity.

Exposure of the acid alcohol extract to the reaction buffers, modified as described (section 2.6.4), had no effect on antibacterial activity. Similarly, no loss of activity was observed in extracts exposed to, but not incubated in the presence of, any enzyme in reaction buffer.

6.2 Affinity chromatography

Antibacterial activity from acid alcohol extracts loaded onto the lectin columns was eluted in the non-sorbed fraction (Table 28). Recovery of antibacterial activity was incomplete - the activity recovered from Con A-Sepharose, Wheat germ lectin-Sepharose and soybean lectin Sepharose was 58%, 83% and 68% respectively. No activity was eluted either by specific sugars or by non-specific conditions. Similar results were obtained following re-exposure of the non-sorbed fraction to fresh lectin. The addition of releasing sugars, either to the acid alcohol extracts or to the non-sorbed fractions, had no effect on the expression of antibacterial activity.

Table 29. Antibacterial activity and slopes of bacterial growth inhibition curves for the low molecular weight antibacterial component of amniotic fluid against a limited spectrum of bacteria.

	B.subtilis	M.luteus	E.coli	P.aeruginosa	S.faecalis	S.aureus	C.perfringens	B.fragilis
Antibacterial activity (units/ml)	23.4 (13.7-40.1)	34.2 (20.6-56.9)	17.3 (8.4-35.8)	16.5 (9.1-30.0)	14.9 (6.0-37.2)	19.2 (9.4-39.2)	7.9 (4.5-13.8)	7.5 (3.8 -14.7)
Slope of inhibition curve (mm/log unit)	7.58±0.24	10.51±0.62	6.78±0.29	12.14±0.48	7.64±0.22	6.58±0.32	9.22±0.61	9.06±0.09

All bacterial species were tested using inocula of 10^6 viable cells/ml. Antibacterial activity is expressed as geometric mean (standard deviation range), while the slopes of the inhibition curves are expressed as arithmetic mean ± 1 s.d..

6.3 Antibacterial spectrum

The antibacterial activity of the acid alcohol extract of lyophilised ultrafiltrate of amniotic fluid was determined against two concentrations of the standard indicator organism, B. subtilis and against a limited spectrum of bacteria.

The mean antibacterial activity displayed by 8 acid alcohol extracts against B.subtilis in assays with inoculum sizes of 10^6 and 10^8 viable cells/ml was 23.4 (13 - 40.1) units/ml and 14.4 (10.1 - 20.4) units/ml, respectively ($P < 0.025$, paired t-test). The corresponding slopes of the growth inhibition curves were 7.58 $\pm$ 0.24 mm/log. unit and 7.48 $\pm$ 0.35 mm/log. unit respectively. The standard inoculum for the test procedure was 10^8 viable cell/ml. For routine use, this concentration was employed as it gave more reproducible results and better zone definition (section 2.2.2). However, for comparison with faster growing bacteria, the reduced inoculum size of 10^6 viable cells/ml was used.

The mean antibacterial activities of 8 acid alcohol extracts against a limited spectrum of bacteria is shown in Table 29. Also shown are the mean slopes of the growth inhibition curves for each bacterial species. The low molecular weight antibacterial component was active against all species tested. The rank order for sensitivity of the species tested was, most sensitive species first; M. luteus > B. subtilis > S. aureus > E. coli > P. aeruginosa > S. faecalis > C. perfringens > B. fragilis. The slopes of the growth inhibition curves ranged from 6.6 mm/log. unit for S. aureus to 12.1 mm/log. unit for P. aeruginosa. Thus it can be seen that the zone diameter produced by the low molecular weight component is not simply due to diffusion but rather a certain number of bacteria (the "critical mass") which vary dependent upon susceptibility of each species.

There was no difference in susceptibilities of Gram-positive as compared with Gram-negative organisms. Nor was there any overall difference in the slope of the bacterial growth inhibition curves. The anaerobic bacteria studied (C. perfringens and B. fragilis) were less susceptible to the antibacterial effects of the acid alcohol extracts than the aerobes tested ($P < 0.0001$).

The possible influence of aerobic incubation conditions was examined. The antibacterial activity of the acid alcohol extracts was reassessed under anaerobic conditions, using those aerobic bacteria which are facultatively anaerobic (i.e. S. aureus, E. coli, and S. faecalis). There was no difference in activity, in terms of either susceptibility or slope of the inhibition curve, of these three bacteria under aerobic or anaerobic conditions.

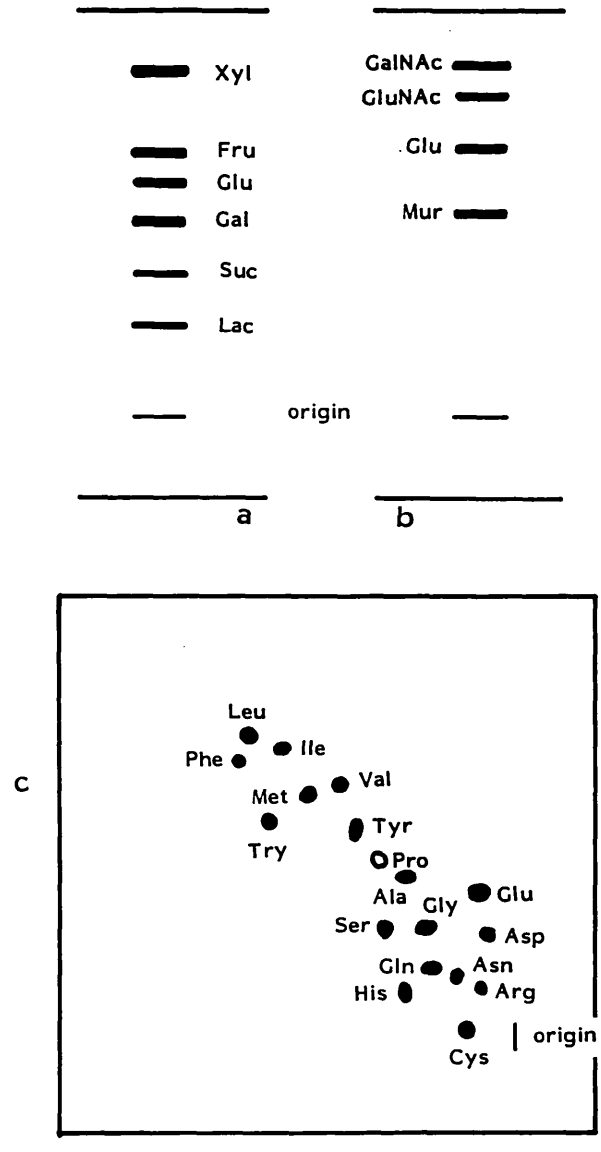


Fig. 23. a) Thin layer chromatography of neutral sugars. Standard aldose and ketose sugars separated on F1500 silica in ethyl acetate : pyridine : acetic acid; Xyl - xylose, Fru - fructose, Glu - glucose, Gal - galactose, Suc - sucrose, Lac - lactose.

b) Thin layer chromatography of amino sugars. Standard amino sugars separated on F1440 cellulose in n-butanol : pyridine : water; GluNAc - N-acetylglucosamine, GalNAc - N-acetylgalactosamine, Mur - muramic acid, Glu - glucose.

c) Thin layer chromatography of amino acids. Standard amino acids separated on F1440 cellulose plates by two dimensional development. 1st solvent; methylpropan-2-ol : butanone : acetic acid : water, 2nd solvent; butanone : acetone : acetic acid : water. Ala - alanine, Arg - arginine, Asn - asparagine, Asp - aspartic acid, Cys - cystine, Gln - glutamine, Glu - glutamic acid, Gly - glycine, His - histidine, Ile - Isoleucine, Leu - leucine, Met - methionine, Phe - phenylalanine, Pro - proline, Ser - serine, Try - tryptophan, Tyr - tyrosine, Val - valine.

6.4 Thin layer chromatography

6.4.1 Sugar chromatography

A representation of a typical chromatograph of neutral saccharide standards is shown in Fig. 23a, the limit of detection was approximately 0.6 nmol. No sugars were detected in either the acid alcohol extracts or the post-HPLC antibacterial fraction (n = 6).

A similar representation is depicted in Fig. 23b, of amino sugar standards, the limit of detection was approximately 0.1 nmol. No amino sugars were observed in either acid alcohol extracts or the purified fraction from HPLC (n = 6).

6.4.2 Amino acid chromatography

A typical two dimensional thin-layer chromatograph of amino acid standards is shown in Fig. 23c. The detection limit for the amino acids was approximately 0.08 nmol. Ultrafiltrates of amniotic fluid gave a similar pattern of amino acids to that normally found in serum, as did lyophilised amniotic fluid ultrafiltrates when diluted appropriately. Acid alcohol extracts were enriched with respect to the more apolar amino acids, while the residual extract was depleted of those amino acids.

Amino acid chromatographs of HPLC fractions collected following separation of the acid alcohol extracts demonstrated free amino acids eluting with the void volume (fraction 1) or just after (fractions 2 & 3). No free amino acids were associated with the antibacterial fraction (6). While no antibacterial activity could be detected following the hydrolysis of HPLC fraction 6, thin layer chromatographs of hydrolysates revealed the presence of free amino acids.

The following amino acids were detected:

Alanine, Glutamate, Glycine, Leucine and Serine

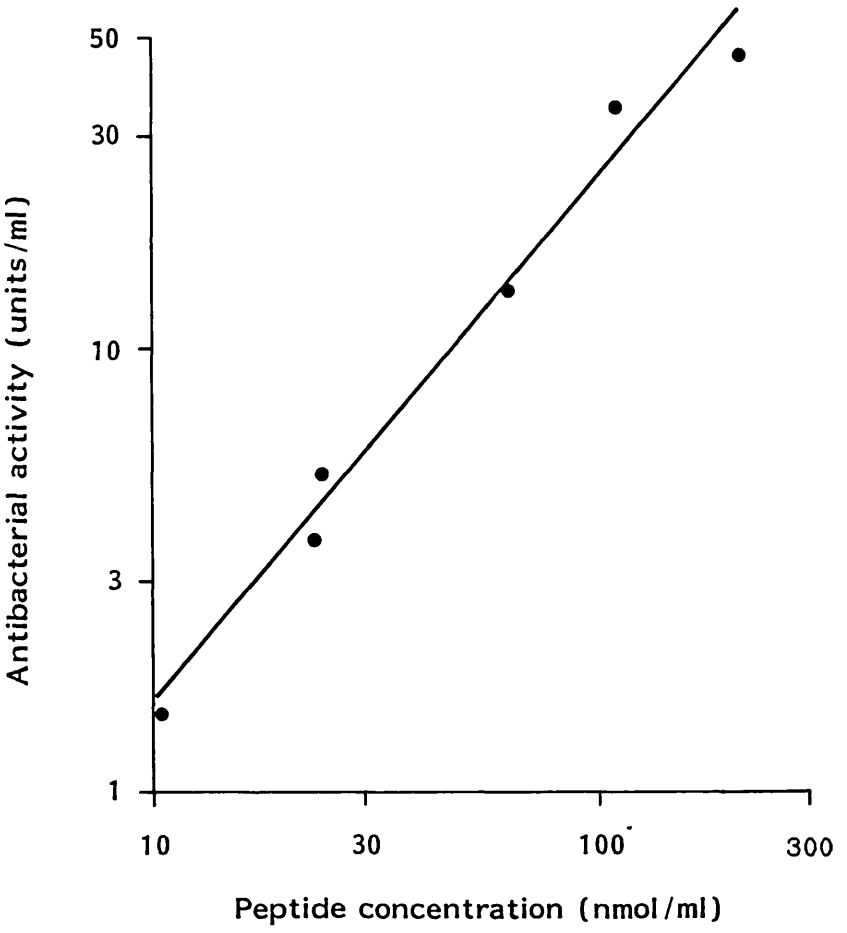


Fig. 24. The relation between estimated peptide concentration and antibacterial activity associated with the active fraction following high performance liquid chromatography of acid alcohol extracts of amniotic fluid ultrafiltrates.

However, under acid hydrolysis, asparagine is converted to aspartic acid and glutamine to glutamic acid. Proline is not detected by the visualisation technique used. The concentrations of these amino acids were estimated semi-quantitatively by TLC in 6 samples of HPLC-fractionated material. The amino acids were present in the following approximate molar ratios: Ala_1, Glu_1, Gly_2, Leu_1, Ser_1. A peptide of this composition would have a molecular weight of 553. The relationship between estimated peptide concentration [P] and antibacterial activity [A] of these samples is shown in Fig. 24. The equation of the regression line was:

$$[A] = -0.014 + 0.24[P], \quad r^2 = 0.93$$

The ratio of antibacterial activity to peptide concentration was calculated for these hydrolysed samples. This gave the relationship of 0.212 ± 0.060 units/nmol as compared with 0.238 ± 0.028 units/nmol derived from the regression equation.

Further analysis of the HPLC fraction, by the peptide laboratory of the Biochemistry Department at Imperial College of Science and Technology, revealed the heterogenous composition of this fraction. The fraction (192 units, approximately equivalent to 800 nmol peptide) was repurified by gradient elution reverse-phase HPLC and the active fraction (19.4 units) subjected to amino acid analysis and fast atom bombardment mass spectroscopy. From the activity of this refractionated HPLC fraction, approximately 82 nmol of peptide would have been expected by interpolation on Fig. 24. However, only approximately 0.6 nmol was detected; Amino acid analysis and FAB mass spectroscopy both suggested a peptide of composition Gln_2, Pro_1, Gly_1, or its isomer Glu_1, Gln_1, Pro_1, Gly_1, with an amidated carboxy-terminus (i.e. $-CO-NH_2$). The specific activity of this peptide was, therefore, 14.4/0.6 = 32.3 units/nmol.

Table 30. Antibacterial activity (units/ml) of synthesised peptides following incubation in the absence or presence of zinc.

Peptide	1	2	3	4	5	6	7	8	9	10	11	12
Absence	-	-	-	-	-	-	-	-	-	-	-	-
Presence	-	-	-	-	-	-	-	-	91.4	-	-	-

The peptides were dissolved in 50 mmol/l ammonium acetate, pH 7.4 at a nominal concentration of 10 mmol/l. After incubation (24 hours, 4°C) in the absence or presence of zinc chloride, the peptides were tested for antibacterial activity to B. subtilis.

6.5 Peptide synthesis

The twelve tetra-peptides listed in Table 5 were synthesised. The yields of these peptides, as assessed by measurement of dry weight, ranged from 58% to 98%; mean yield (± 1 s.d.) was 74.7% ± 12.4 %. Assessment of purity, by thin layer chromatography, revealed the absence of any major peptide products other than the apparent parent tetra-peptide. However, further confirmations of synthesis, e.g. by test hydrolysis, were not performed. Using starting concentrations of 10 mmol/l, the peptides were tested for activity to B. subtilis.The results of the antibacterial assays are shown in Table 30. Activity was detected only in Peptide 9 (Gln-Pro-Gly-Gln), following incubation in the presence of zinc. Simple addition of zinc chloride prior to testing did not result in production of antibacterial activity. Concentrations greater than 100 µmol/l exerted an inhibitory effect. Extrapolation of the zone diameter vs. log.(peptide) dilution curve in the usual manner resulted in an estimate for antibacterial activity of 90.6 units/ml. After allowing for variance from the stated concentration of peptide (actual concentration 10.6 mmol/l) due to slight differences in recovery, this resulted in a value for DF_o of 116 µmol/l, i.e. the concentration at which it would be expected that the antibacterial activity would be diluted to zero was 116 µmol/l (= 49.6 µg/ml). The specific activity of this synthetic peptide was, therefore, 90.6/10.6 = 8.6 units/µmol. The slope of the antibacterial assay was 6.4 mm/log. dilution, as compared with that of the low molecular weight antibacterial component from amniotic fluid, of 7.6 ± 0.55 mm/log. unit ($P < 0.001$).

The active peptide was fractionated by reverse phase HPLC using gradient elution. The chromatograph recorded at 215 nm is shown

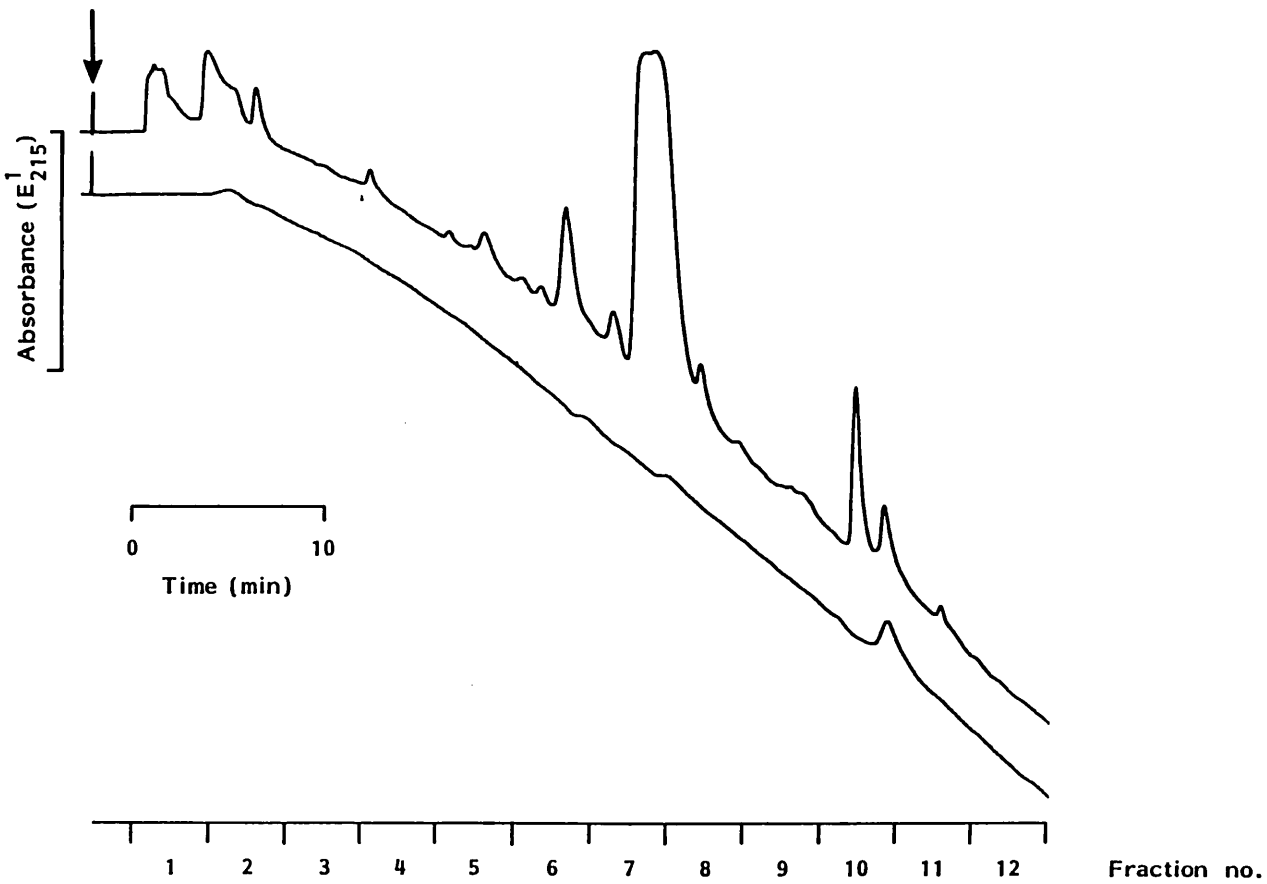


Fig. 25. Fractionation of peptide product 9 by gradient elution reverse phase HPLC. The column (Zorbax ODS, 4.6 x 250 mm) was equilibrated at 1.0 ml/min with 50 mmol/l ammonium acetate, pH 7.0. The synthetic antibacterial peptide (1.0 ml) was loaded onto the column (arrow) and a linear gradient extending from 0 to 100% methanol simultaneously applied. The absorbance of the eluent, which was collected in 4.0 ml fractions, was monitored at 215 nm (upper trace). It was compared with that obtained from a blank elution (lower trace). The antibacterial activity of each 4 ml fraction was determined.

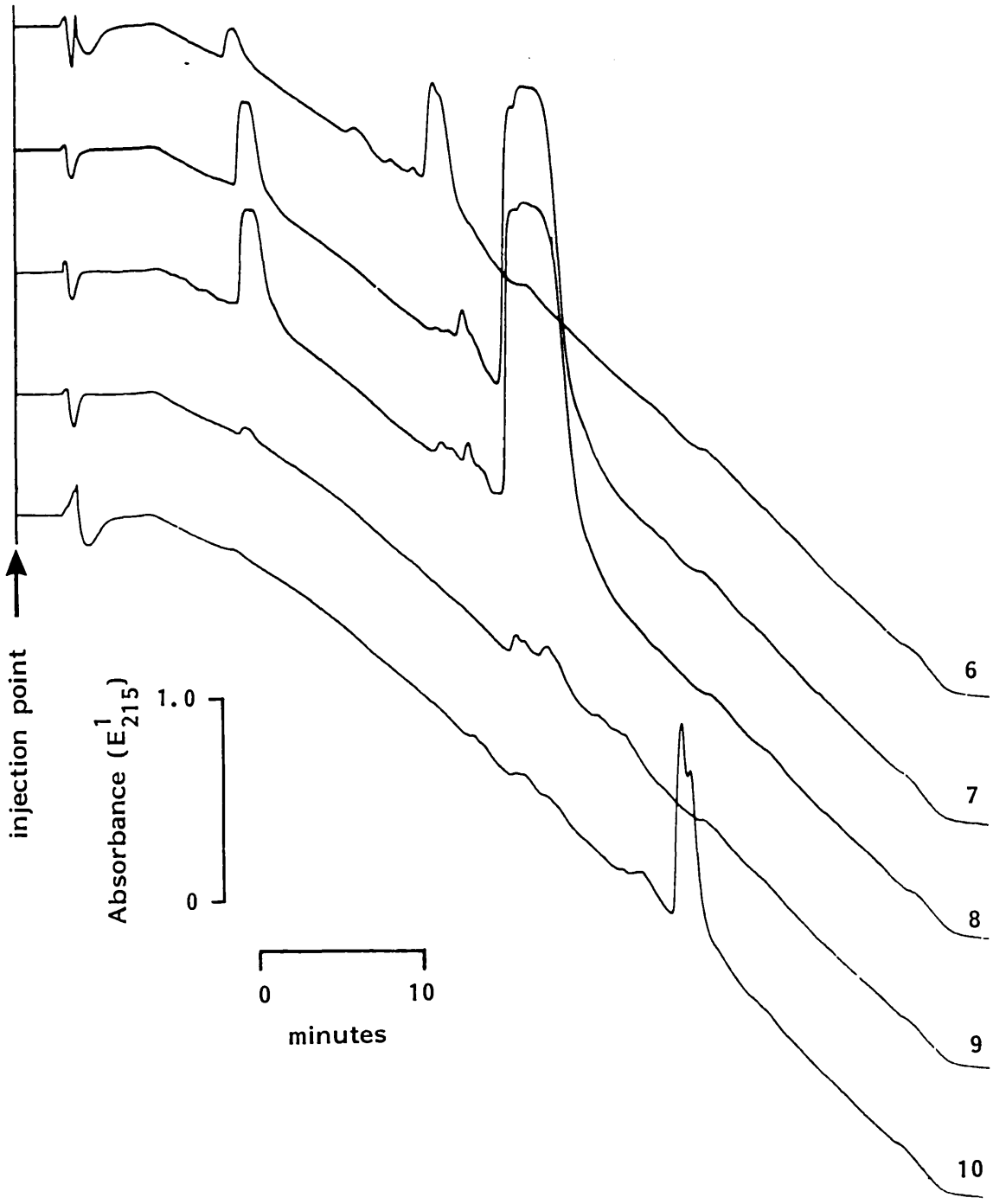


Fig. 26. Rechromatography of HPLC fractionated synthetic peptide. The fractions indicated were obtained from the separation illustrated in Fig. 25. They were rechromatographed under conditions identical to those used previously. Antibacterial activity was detected in fractions 7 and 8 only.

in Fig. 25. Twelve 4 ml fractions were collected as indicated, assayed for antibacterial activity and re-chromatographed (Fig. 26). Antibacterial activity was recovered only in fraction 7 and 8, which is the same as that obtained following gradient elution reverse phase HPLC of the low molecular weight component in acid alcohol extracts (cf. Fig. 16).

6.6 Discussion

Superficially, the results of investigation of the effects of enzyme solutions, on expression of low molecular weight antibacterial activity appear to be relatively uninformative. The resistance of the amniotic fluid component to trypsin and chymotrypsin is in agreement with previous data (Schlievert et al. 1976c), as is the susceptibility to attack by carboxypeptidase. This at least suggests that the low molecular weight antibacterial factor has a peptide component.

Only proteins and peptides containing basic and aromatic amino acids tend to be cleaved by trypsin and chymotrypsin respectively (Smyth 1967, Desnuelle 1960) which suggested that these types of amino acid were absent from the low molecular weight antibacterial component. Carboxypeptidase is only able to act on peptides and proteins with an unrestricted carboxyl terminus (Neurath 1960). The limited action of staphylococcal V8 protease on the expression of antibacterial activity reveals a great deal of information on the structure of the antibacterial peptide. Under acid conditions, this peptidase only cleaves across the carbonyl junction of a glutamyl bond (Houmard & Drapeau 1972). In addition, all Glx-bonds are readily cleaved except for the configuration, -Gln-Pro-, which is only hydrolysed very slowly. Lack of effect of incubation of the extract with amylase or glycopeptidase tended to suggest lack of a sugar moiety in the structure of the low molecular weight antibacterial component.

It had been proposed, in a commentary, that the low molecular weight antibacterial component of amniotic fluid ultrafiltrates bound to a lectin column (Stern 1981). This would suggest the presence of a sugar residue. The results observed here indicate that loss of

activity from solutions exposed to lectin does not constitute binding, as no activity was recovered using specific releasing sugars. It was possible that the acid alcohol extract could contain sugar moieties that would bind preferentially to the lectin, thus preventing adsorption of a glycopeptide, from the extract. No sugars were detected by thin layer chromatography in either acid alcohol extracts or the purified, post-HPLC fraction. Nor was any activity bound from the lectin wash solution, that was re-exposed to fresh lectin.

No sugars, in either neutral or amino forms were detected by thin layer chromatography. However, thin layer chromatography for free amino acids revealed their presence in amniotic fluid ultrafiltrates, acid alcohol extracts and the void volume following HPLC, but not in the HPLC fraction with antibacterial activity. Hydrolysis of this fraction revealed the presence of 5 amino acids which were present in the approximate molar ratio which could indicate the presence of a hexapeptide. This does not, of course, indicate that such a hexapeptide was present. Many other possibilities also exist, not the least of which was the possibility that the antibacterial peptide (indicated by the enzymology data) was present in very low concentrations that were masked by the presence of greater concentrations of co-chromatographic peptide material. This does, at least, support the proposition of Sachs & Stern (1979) which suggested the presence of a "family of peptides" in this amniotic fluid fraction.

The activity of the synthetic peptide was much lower than that of the native peptide. Additionally, the slopes of the growth inhibition curves were dissimilar. It was, therefore, unlikely that the structure of the synthetic peptide product was homologous with that of the native

peptide. Although analysis by thin-layer chromatography had indicated that only one peptide product was present, the absolute configuration was not verified. Subsequent investigations have indicated that the synthetic product may have been a penta-peptide. Mass-charge considerations* have suggested the following structure: Gln-Gln-Pro-Gly-Gln, i.e. Peptide 9 with an additional Glutamine residue attached to the N-terminus. Therefore, despite incorporating the intended structure, full expression of activity was not displayed. Further work is obviously required: the tetra-peptide should be re-synthesised and its structure confirmed prior to investigation of antibacterial activity.

*(Carried out after completion of the project, by Dr. J. Halkett (personal communication)).

CHAPTER SEVEN

Source of the low molecular weight antibacterial component of amniotic fluid

The antibacterial activity of human amniotic fluid is extensively documented. Schlievert et al. (1976c) demonstrated that amniotic fluid contained a low molecular weight antibacterial component. It has been shown (Chapter 4) that the low molecular weight component is present in amniotic fluid, in a wide range of concentrations: some samples display activity on initial testing, others require (twenty-fold) concentration of the sample to produce levels detectable by the agar diffusion assay. The source of the low molecular weight antibacterial component has not been investigated. It was therefore of interest to determine the distribution of this factor within the pregnant uterine cavity.

Table 31. Distribution of antibacterial activity from placenta and fetal membranes.

	Amnion	Chorion	Placenta
Homogenate	ND	ND	1.3 ± 0.67
Cell debris	ND	ND	ND
Fatty layer	ND	ND	ND
Alcohol extract	ND	ND	0.73 ± 0.24

Antibacterial activity expressed in units/g tissue. Mean values ± 1s.d. (N = 4). Limit of detection 0.08 units/g tissue. ND - not detected.

Table 32. Distribution of antibacterial activity through subcellular fractions of human placental homogenates.

Fraction	Activity (units/g tissue)	(%)	Specific activity units/mg protein	ratio	Slope of inhibition curve (mm/unit)
Homogenate	5.08 ± 0.63	(100)	0.19		
Cytosol	1.80 ± 0.40	36	0.13	1.0	7.5 ± 0.32
Nucleus	0.92 ± 0.19	18	0.37	2.8	7.5 ± 0.75
Mitochondria	0.77 ± 0.16	15	0.52	4.0	5.7 ± 0.42
Microsomes	1.79 ± 0.90	35	4.33	33.2	5.7 ± 0.64

Antibacterial activity expressed as a fraction of both tissue weight (units/g tissue) and the protein concentration of each subcellular fraction.
Mean values ±1 s.d. (n = 3).

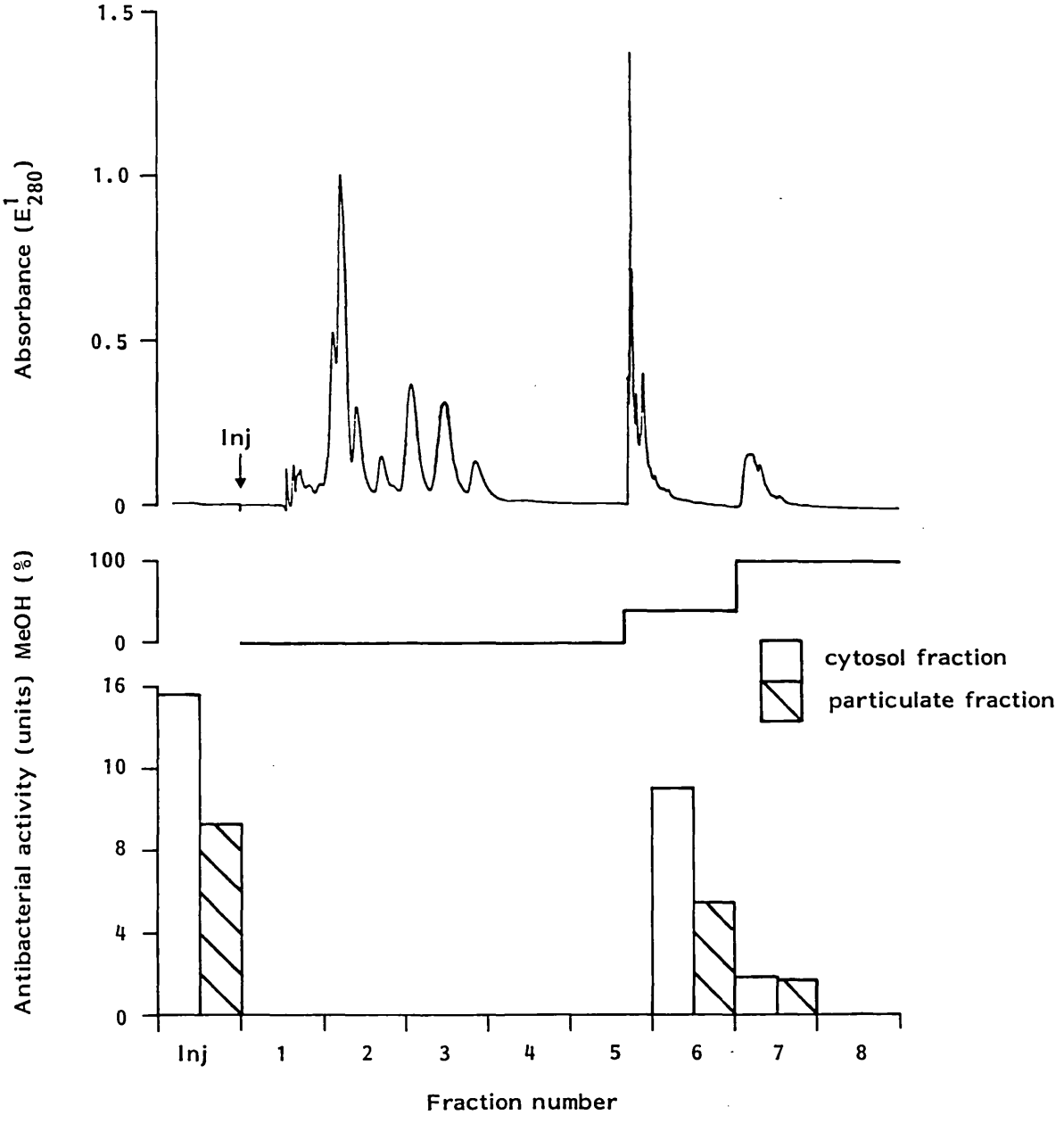


Fig. 27. Separation of low molecular weight components of extracts of both the combined mitochondrial and microsomal fractions (particulate) and the cytosol of human placental homogenates by reverse phase HPLC and subsequent distribution of antibacterial activity. Extracts were injected onto the column (Zorbax ODS, 4.6 x 250 mm) and eluted at 1.0 ml/min with an echelon pattern of 50 mmol/l ammonium acetate, pH 7.0 to 40% and 100% methanol (centre panel). The absorbance of the eluate was monitored at 280 nm in a 1.0 cm flow-through cell (upper panel) and the antibacterial activity of each 4 ml fraction determined (lower panel).

7.1 Investigation of low molecular weight fractions of placenta, amnionic and chorionic membranes for antibacterial activity

7.1.1 Tissue homogenates and sub-cellular fractionation

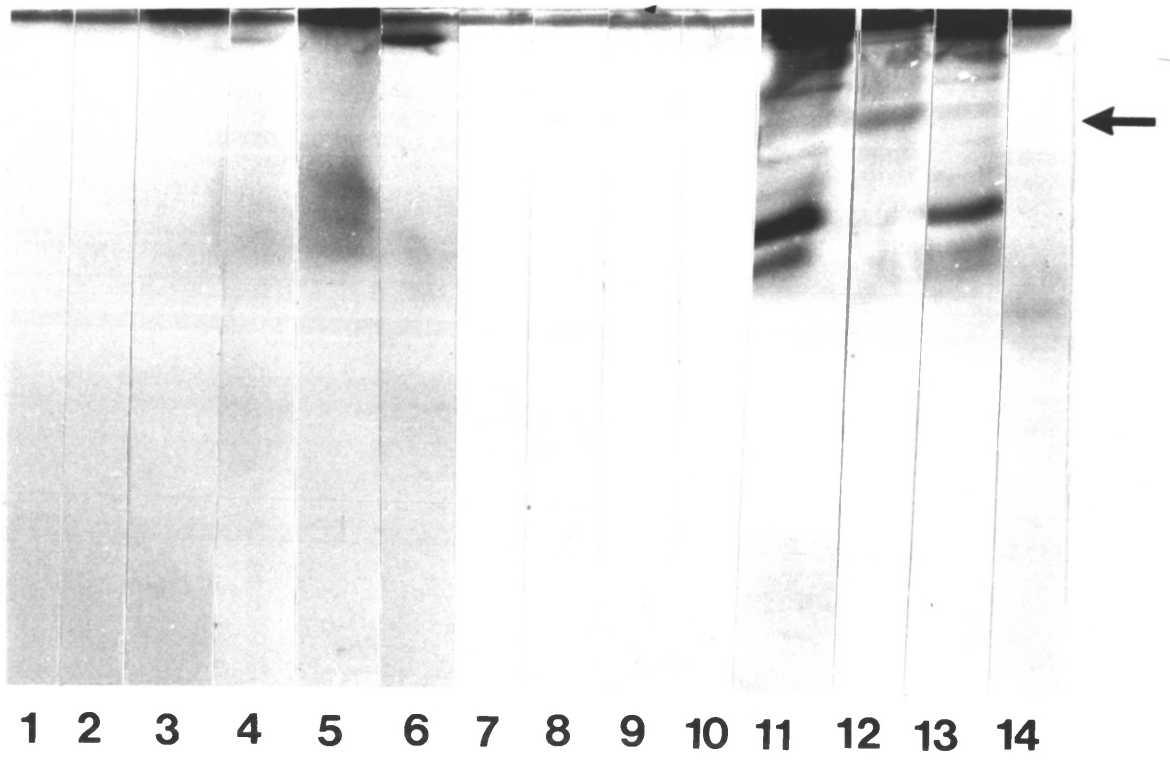
Antibacterial activity was observed in all homogenates of placental villous tissue (Table 31), but no activity was detected in homogenates of either amnion or chorion. No activity was found in the cell debris or fatty layer. Subsequently, 60% of the activity expressed by the placental tissue homogenate was recovered in the acid alcohol extract of the lyophilised ultrafiltrate (Table 31). No activity was recovered in similar extracts of homogenates of amnion or chorion.

Sub-cellular fractions were prepared from placental tissues to determine the distribution of antibacterial activity within the cell. Antibacterial activity was present in all sub-cellular compartments, but a larger proportion was found in the cytosolic and microsomal fractions (Table 32). When the activity was expressed in relation to the protein content of each fraction, the greater specific activity was found in the microsomal fraction. The corresponding value for amniotic fluid was 2.7 units/mg protein. The gradients of the antibacterial assays of the cytosolic and nuclear fractions were steeper than those of the mitochondrial and microsomal fractions (7.4 ± 0.52 mm/log. unit and 5.7 ± 0.48 mm/log. unit respectively; $P < 0.005$). However, they were comparable to that observed with the low molecular weight antibacterial component found in amniotic fluid (7.6 ± 0.55 mm/log. unit).

7.1.2 High performance liquid chromatographic purification

The elution profile of the combined mitochondrial and microsomal acid alcohol extracts ('Particulate' fraction) following reverse phase HPLC is shown in Fig. 27, together with that of the

Fig. 28. One dimensional SDS- polyacrylamide gel analysis of acid alcohol extracts.



Acid alcohol extracts were prepared from placental tissue fractions and reverse phase HPLC fractions. The protein composition of these fractions was analysed by one dimensional SDS- polyacrylamide gel electrophoresis followed by silver staining.

Key : acid alcohol extracts prepared from

1. Nuclear fraction
2. Mitochondrial fraction
3. Microsomal fraction
4. Cytosolic fraction
5. Concentrated, combined mitochondrial & microsomal fractions
6. Concentrated cytosol fraction
7. HPLC #1
8. HPLC #2
9. HPLC #3
10. HPLC #4
11. HPLC #5
12. HPLC #6
13. HPLC #7
14. HPLC #8

The arrow indicates the protein species which contained the maximal amount of antibacterial activity.

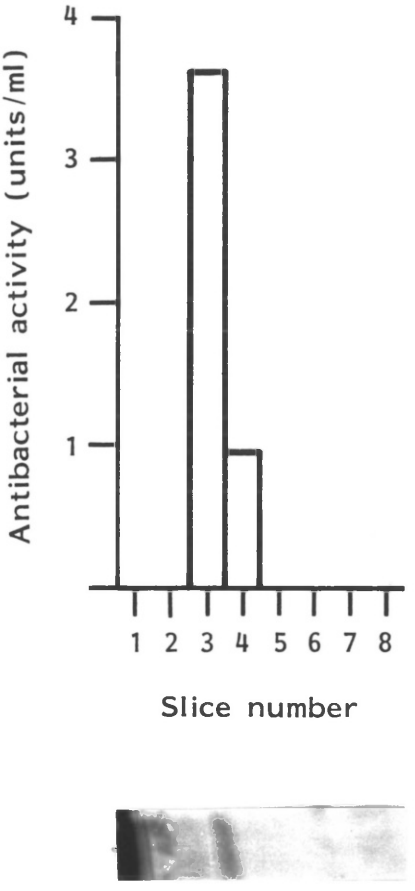


Fig. 29. Recovery of antibacterial activity from gel slices following PAGE analysis of placental tissue extracts.

The gel lane containing HPLC fraction 6 (lane 12, Fig.28) was excised from the polyacrylamide gel and cut into 0.5 cm slices. Each slice was destained separately and assayed for antibacterial activity to B. subtilis.

cytosolic acid alcohol extract (cf. elution profile of amniotic fluid extract, Fig. 11). The components possessing antibacterial activity were resolved into two discrete fractions. The recovery of activity following chromatography of the extracts of the cytosolic and the combined particulate fraction was 81% and 75% respectively.

7.1.3 Gel electrophoretic analysis

Acid alcohol extracts of the separate sub-cellular fractions were analysed by polyacrylamide gel electrophoresis (Fig. 28). Silver staining of the gel following electrophoresis of the cytosolic extract revealed the presence of at least one small peptide. In contrast, electrophoretic analysis of the extracts of the nuclear, mitochondrial and microsomal fractions showed no visible peptides after silver staining. The combined mitochondrial and microsomal extracts were concentrated and subjected to PAGE analysis. Silver staining revealed the presence of those distinct molecular species (Fig. 28).

The eluted fractions from HPLC of the combined particulate extract were also analysed by gel electrophoresis (Fig. 28). Silver staining revealed the heterogenous composition of peptides present in fraction 5 and one major and several minor peptides in each of fractions 6 and 7 respectively. No peptides were apparent in fraction 1, 2, 3, 4 or 8. A similar electrophoretogram was obtained following analysis of the cytosolic extract.

Since most of the eluted activity was found in fraction 6, extracts from 5 mm gel slices excised from the lane bearing this fraction were assayed for antibacterial activity (Fig. 29). The activity was found to be confined to the two gel slices which contained the most abundant peptide as determined by silver stain. The gel lane containing fraction 6 of the HPLC of the cytosolic

extract was similarly treated, with similar results.

7.2 Discussion

The antibacterial activity of human amniotic fluid is extensively documented, most recently by Schlievert et al. (1977) and Hawkins and Scane (1983). The major antibacterial components of amniotic fluid are the protein B-lysin (Ford et al. 1977, 1981b) and a low molecular weight peptide first described by Schlievert et al. (1976c). There are also small amounts of other antibacterial proteins (immunoglobulins, leukin, lysozyme, peroxidase and transferrin). No relationship has been demonstrated between antibacterial activity of whole amniotic fluid and the concentrations detected of these other proteins (Feingold et al. 1979, Ford et al. 1981a, Thadepalli et al. 1982, Blanco et al. 1983a). On initial testing only one in four amniotic fluid samples displayed antibacterial activity due to the low molecular weight peptide (section 4.1.2). Following concentration of these samples, activity was found in all of these fluids.

It was therefore of interest to determine the distribution of this factor within the pregnant uterine cavity. Antibacterial activity was only detectable in the placenta and not in amnion or chorion. Further examination revealed antibacterial activity due to low molecular weight substances in all subcellular fractions, but in greatest proportion in the cytosolic and microsomal fractions. The activity of the latter, expressed in relation to the protein concentration, was similar to that of amniotic fluid. Analysis of these placental fractions by gel electrophoresis suggested that the antibacterial component was either the same molecular species present in all the sub-cellular fractions or a series of substances with very similar molecular properties. In contrast, antibacterial activity could not be detected in the chorionic and amnionic membranes, which suggested that the low molecular weight antibacterial substance does

not cross extra-cellular membranes readily.

The nuclear envelope contains pores (approximately 65 nm in diameter) and, in many cells, bears ribosomes in continuity with the endoplasmic reticulum found in the cytoplasm. Consequently, many proteins synthesised in the cytoplasm can travel quite freely into the nucleus without the need for translocation (Watson 1955, Lockwood 1971). The low molecular weight antibacterial component is likely to be freely distributed between the nucleus and the cytoplasm. In accordance with this, the slope of the growth inhibition curve of the nuclear component was the same as that of the cytosolic component.

Mitochondria possess both inner and outer membranes, which are not contiguous. Although a small number of hydrophobic polypeptides are synthesised from mitochondrial DNA (Schatz 1979), most mitochondrial polypeptides are encoded by nuclear DNA and are synthesised as larger precursor molecules on cytoplasmic polyribosomes. These precursor molecules possess an amino-terminal extension, which interacts with a mitochondrial receptor to allow translocation of the precursor polypeptide into the mitochondrion. Processing to the mature form may either occur during translocation (Raymond & Shore 1979) or once within the mitochondrial matrix (Zwizinski & Neupert 1983). The recognition sequence is an extension, ranging from 500 to 6000 daltons, of a multikilodalton protein (Maccecchini et al. 1979, Chien & Freeman 1984) and not of a small peptide. The presence of the antibacterial molecule in the mitochondrial fraction was thus intriguing, and could be a serendipitous by-product of protein processing.

The microsomal fraction prepared in this study composed of a heterogeneous population of intracellular membranous structures, predominately involved in either transmural transport or the

synthesis of membranous and secretory proteins (Svardal & Pryme 1980). These secretory proteins contain a hydrophobic amino-terminal leader sequence of 15 - 30 amino acids (Shields & Blobel 1977) which promotes the attachment of nascent polypeptides to microsomal membranes during synthesis on polyribosomes (Walter & Blobel 1981a,b). The pre-secretory proteins synthesised are transported across the membrane into the intra-cysternal space of the endoplasmic reticulum, where the leader sequence is cleaved and post-translational modifications ensue (Jackson & Blobel 1977).

Since the antibacterial activity and the specific activity of the microsomal fraction were much greater than in the other subcellular fractions, it was possible that the low molecular weight component was stored in intracellular microsomal vesicles, possibily for extra-cellular secretion. The slope of the growth inhibition curve of this fraction was lower than that of the cytosolic and nuclear fractions. This may have been due to post-translational modification and may therefore represent a less active and more stable storage form. These propositions supported the possibility that the placenta was the source of the low molecular weight antibacterial component found in amniotic fluid.

In summary, antibacterial activity very similar to that expressed by human amniotic fluid has been detected in all sub-cellular fractions of the human placenta, where it presumably functioned to protect the body of the placenta from microbial attack. The low molecular weight component was present in very large quantities in the placental microsomal fraction, indicative of the possibility of it being secreted into the amniotic fluid space, where it may perform the same function.

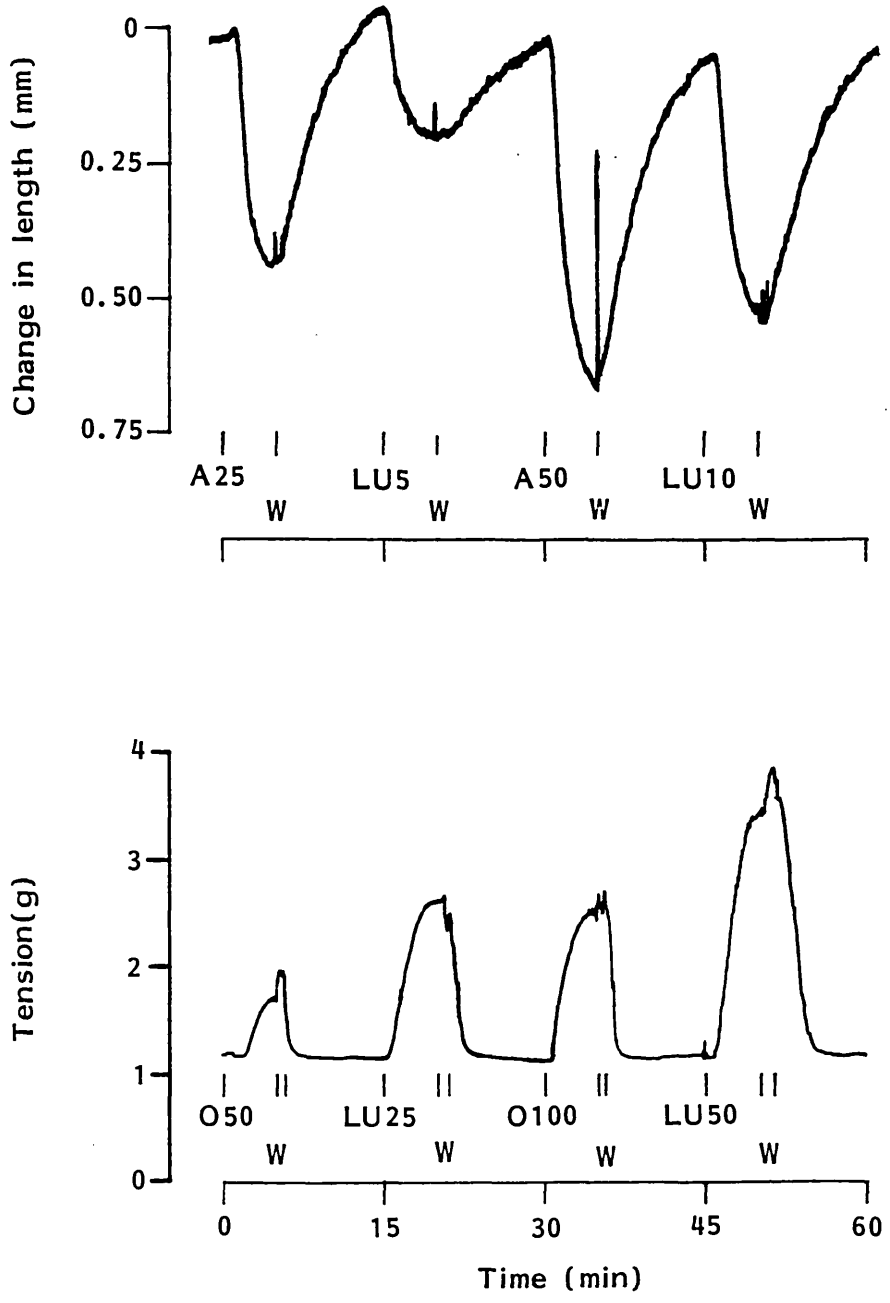


Fig. 30. a) Isotonic comparison of the bronchodilator actions of adrenaline and the low molecular weight fraction of amniotic fluid on the isolated guinea-pig tracheal chain preparation suspended in Krebs-Henseleit's solution at 37°C.
A - Adrenaline (dose in ng)
LU - Lyophilised ultrafiltrate (dose in mg)
W - wash

b) Comparison of the oxytocic actions of oxytocin and the low molecular weight fraction of amniotic fluid on the isolated guinea-pig uterine horn preparation suspended in de Jalon's solution at 37°C under isometric conditions.
O - Oxytocin (dose in μunits)
LU - Lyophilised ultrafiltrate (dose in μg)
W - wash

CHAPTER EIGHT

General Pharmacology

Amniotic fluid fractions previously described were investigated pharmacologically. The contractile effect of these solutions on the three uterine tissue preparations were standardised against oxytocin. In a similar manner, the relaxatory effect on the guinea-pig tracheal chain preparation was standardised against adrenaline. These drugs were used as the reaction curves were similar i.e. a) a similar time from dosing to onset of action,

b) the log. dose response curves were similar in magnitude and shape

c) analysis of variance revealed no significant difference in parallelism from 2 + 2 bioassay.

Accordingly, it was possible to "calibrate" isolated organ preparations in terms of the response to the pharmocological standard. The pharmacological activities of amniotic fluid fractions were estimated by comparison of the response to two given doses of unknowns, bracketed around responses of two different doses of oxytocin or adrenaline respectively, using joint linear regression analysis (Schild 1942). Sample results of such experiments are shown in Figs. 30a and 30b. One oxytocic unit was arbitarily deemed to be equivalent to that effect exerted by 1 munit of oxytocin, while 1 bronchodilator unit was deemed to be equivalent to that exerted by 1 ng adrenaline. Most of the pharmacological investigations were performed on either the isolated guinea-pig uterine horn or the guinea-pig tracheal chain.

The actions of the low molecular weight fraction on smooth

muscle tissue was examined by two main approaches. Firstly, the distribution of bronchodilator and oxytocic activities was examined through the purification sequence described. Secondly, the pharmacological profiles of the two actions were investigated together with relevant biochemical manipulations.

8.1 Pharmacological activity of amniotic fluid fractions.

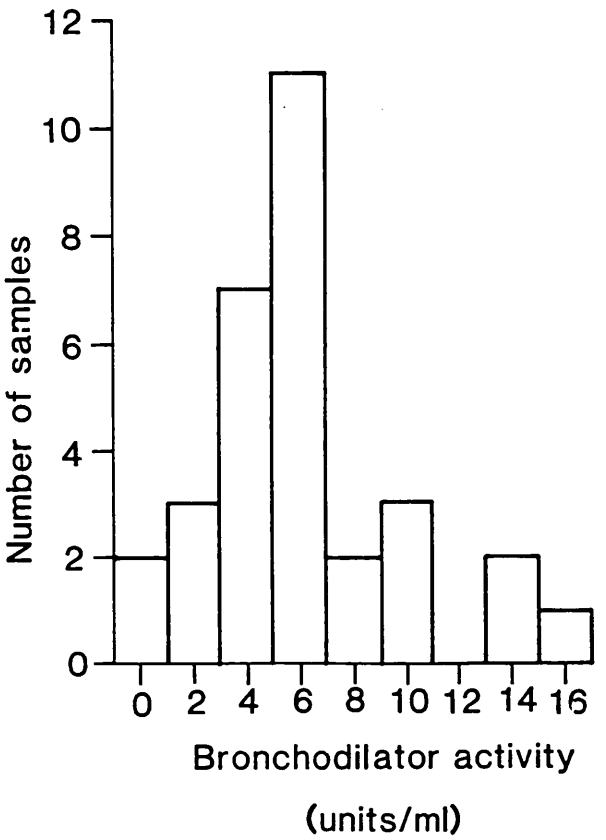
8.1.1 Lyophilates of amniotic fluid ultrafiltrates.

Oxytocic and bronchodilatory activity was assessed in samples of lyophilised ultrafiltrates of amniotic fluid. The lyophilates were tested at 10-fold or 20-fold concentrations (v/v), relative to the original ultrafiltrate. The concentrations quoted below are weight lyophilate per ml bath volume. Amniotic fluid ultrafiltrates contained 10.9 ± 0.52 mg/ml solutes (Table 18), while the bath volume was 6.0 ml. Thus 100 ul of a 20-fold concentrated amniotic fluid ultrafiltrate contained approximately 22 mg of suspended lyophilate, which would produce a resultant concentration in the organ bath of approximately 4 mg/ml.

At low concentrations (4 - 20 µg/ml), the lyophilates exerted an action equivalent to 4.2 ± 3.0 oxytocin units on the guinea-pig uterine horn preparation. At higher concentrations (1 - 1.5 mg/ml) a slight relaxatory action was observed. On the guinea-pig tracheal chain, the lyophilate exerted no effect at low concentrations. Higher concentrations (1 - 2 mg/ml) produced a marked bronchodilator action (1.0 ± 0.8 units/mg).

8.1.2 Lyophilates of gel filtration products.

The smooth muscle actions of lyophilised ultrafiltrates were assessed following separation on Sephadex G10 (section 2.5.4). The tubes associated with each major UV-peak were pooled, lyophilised and tested for oxytocic and bronchodilator activity. Both bronchodilator



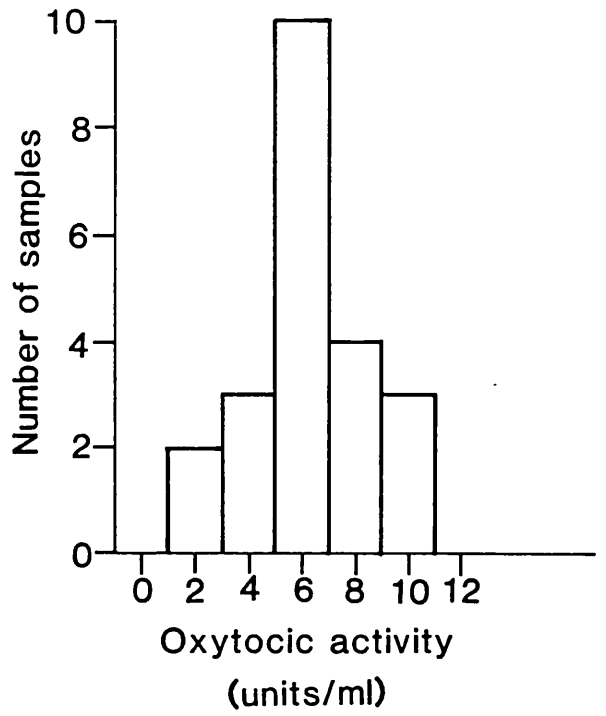


Fig.31. The distribution of the bronchodilator and oxytocic activities of the fraction (III) resulting from gel filtration of lyophilised ultrafiltrates of amniotic fluid.
1 unit of bronchodilator activity was deemed to be equivalent to the effect exerted by 1 ng adrenaline, while 1 oxytocic unit was deemed to be equivalent to the effect exerted by 1 μunit oxytocin.

and oxytocic activities eluted associated with the major absorbent peak (III), i.e. in a position equivalent to that for the elution of antibacterial activity. The bronchodilator activity of the G10 fraction was 5.9 ± 3.7 adrenaline units/mg, while the smooth muscle stimulant activity was present at 6.3 ± 2.2 oxytocic units/mg. The distributions of these activities are shown in Figs. 31a and 31b. The bulk of the weight (56 ± 11%) of the lyophilised ultrafiltrates loaded onto the G10 column was also eluted in the pooled fraction comprising the peak with maximal UV adsorption.

The oxytocic activity of the gel filtration product was also assessed in samples of amniotic fluid obtained from patients induced for reasons other than post maturity. The activity of the samples from normal (post-dates) samples (6.3 ± 2.2 units/mg) was similar to those from diabetic patients (6.3 ± 3.5 units/mg), patients with intra-uterine growth retardation (4.3 ± 2.1 units/mg) and those with hypertension (6.9 ± 1.7units/mg). Samples obtained from toxaemic patients exerted a greater oxytocic action (14.4 ± 4.2 units/mg, $n = 3$, $P < 0.01$).

8.1.3 Lyophilates of HPLC fractions

The bronchodilator and oxytocic activities were assessed in the HPLC fractions following separation of the Sephadex G10 gel filtration product. Bronchodilator activity was eluted in fraction 1 (Fig. 11), while oxytocic activity was eluted in fractions 4 and 6.

Bronchodilator activity equivalent to 5.1 ± 2.4 units/mg was detected in the void fraction (1). In contrast, elution of low molecular weight components with oxytocic activity from the HPLC column was retarded. Two distinct zones of such activity were detected, the first "polar" component eluted in fraction 3 (Fig. 11), while the second "apolar" component eluted in fraction 6 (Fig. 11).

Table 33. Purification of oxytocic activity from the low molecular weight fraction of amniotic fluid.

Fraction	Weight (mg)	Specific activity units/mg	Yield (units)	Recovery (%)	Purification factor
Lyophilised ultrafiltrate	400	4.2 ± 3.0 (n=7)	1684	(100)	(1)
Gel filtration product	226	6.3 ± 2.1 (n=22)	1405	83	1.5
HPLC #3	7.0	84 ± 34 (n=4)	588	35	19
#6	0.9	789 ± 58 (n=4)	742	44	187

Oxytocic activity is expressed as means ± s.d. (n).

However, the eluate examined for oxytocic activity was usually collected in 1 ml fractions rather than the 4 ml fractions used in the study of antibacterial activity. The apolar component with oxytocic activity was then eluted ahead of the component with antibacterial activity. The fraction containing the antibacterial component exerted neither stimulatory nor relaxatory effects on either the tracheal chain or uterine horn preparations. The mean oxytocic activity in 4 samples of the polar HPLC fraction was 84 ± 34 units/mg, while that of the apolar (HPLC) fraction was 729 ± 58 units/mg. Pharmacological investigation of the nature of the low molecular weight oxytocic action of amniotic fluid was confined to use of the polar and apolar fractions resulting from HPLC. The purification of oxytocic activity is summarised in Table 33.

8.2 Investigation of the pharmacological identity of the bronchodilator and oxytocic actions of low molecular weight amniotic fluid components.

8.2.1 Bronchodilator activity

As can be seen from section 8.2.3, bronchodilator activity of material purified by size exclusion chromatography could not be purified further by HPLC. The fraction containing the bronchodilator activity also contained the inorganic salts. Therefore, a comparison was made between the bronchodilator activity of this fraction, and two solutions of concentrated ionic salts. The first solution (1) contained salts (Na^+, K^+, Mg^{++}, Ca^{++}, Cl^-) in the same concentrations as those detected in the HPLC fraction. Solution 2 contained sodium chloride at a concentration that was iso-osmotic with that of the bronchodilator HPLC fraction.

The bronchodilator activity of HPLC fraction 1 (5.1 ± 2.4 units/mg) was equivalent to that of solution 1 (4.7 ± 1.1

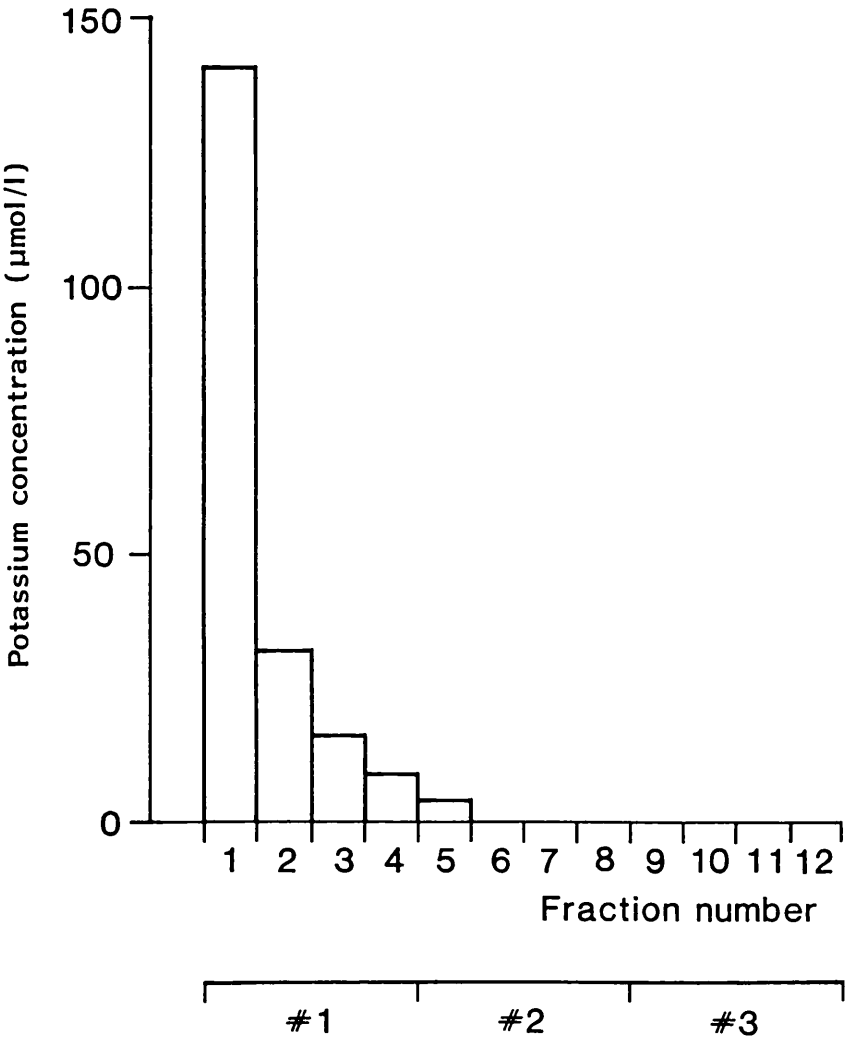


Fig.32. The concentration of potassium was assessed in the eluent following high performance liquid chromatography of lyophilised ultrafiltrates. The correspondence of the 1 ml fractions to those in Fig.11 is indicated by the hashed figures.

units/mg; $0.7 > P > 0.6$) on the basis of 4 estimations. The bronchodilator activity of the iso-osmotic solution (2) was 3.7 ± 2.4 units/mg.

8.2.2 Oxytocic activity

As shown in Table 33, the aqueous, polar and apolar, oxytocic components of the low molecular weight fraction of amniotic fluid were highly purified by HPLC. These were used in pharmacological investigations.

In view of the bronchodilator effects of the ionic components, the concentration of potassium ions was assessed in the eluent following chromatography of the lyophilised ultrafiltrate. The results are shown in Fig. 32. Maximal concentrations of K^+ were detected in fractions containing voided material while the concentrations were below the limit of detection in those fractions possessing oxytocic activity.

The smooth muscle stimulant activity of both the polar and apolar fractions was not mediated by cholinergic stimulation. Equipotent uterine stimulant doses of the low molecular weight oxytocic components and carbamoyl choline (Carbachol, 5 µmol/l) were assessed in the presence of atropine (0.1 µmol/l). The response to carbachol was abolished while the responses to both the polar and apolar uterine stimulants were unaffected. A higher concentration of atropine (3 µmol/l) had no effect on oxytocic activity. The stimulatory effects of tetramethyl ammonium (500 µmol/l) were blocked by tetra-ethyl ammonium (2 mmol/l) and atropine (0.1 µmol/l). The uterine stimulant actions of the polar and apolar molecular species were unaffected.

The uterine stimulant effects of the two HPLC fractions were also assessed in comparison with histamine. Equipotent doses were

assessed following exposure of the isolated organ preparation to mepyramine malate (0.5 µmol/l). The response to a previously equiactive concentration of histamine (2 µmol/l) was abolished in the presence of mepyramine while there was no difference in the response to the oxytocic amniotic fluid components.

It was demonstrated additionally, that the oxytocic responses of the polar and apolar HPLC fractions were not attributable to a tryptaminergic response. Methysergide (5 nmol/l) had no effect on the oxytocic effects of the HPLC fractions, but abolished the response to an equiactive concentration of 5-hydroxytryptamine (50 nmol/l).

It was also demonstrated that the oxytocic response produced by either of the two HPLC fractions, was not due to a direct prostaglandin effect. The oxytocic components were not extracted into ethyl acetate from acidified solutions (50 mmol/l citric acid) of the HPLC fraction, a process which extracted 93% of added radioactive prostagladin E_1. Similarly, the activity was not affected by exposure to pH 12 for 1 hour at 4°C (untreated fraction 650 ± 56 units/ml, treated fraction 740 ± 103 units/mg; n = 4), a procedure which abolished the oxytocic response produced by PGE_1.

Low concentrations (<1 nmol/l) of the SRS-A antagonist, FPL 55712 had no effect on the activity of the apolar HPLC fraction with an oxytocic action. Higher concentrations (between 10 and 100 nmol/l) caused 13% and 68% reductions in activity, while concentrations greater than 1 µmol/l completely abolished the oxytocic response. The oxytocic response to the polar uterine stimulant was unaffected by the presence of FPL 55712, even in concentrations as high as 10 µmol/l.

Pre-treatment of guinea-pig uterine horns with indomethacin

(10 umol/l) caused a significant decrease in contractile response to low doses of oxytocin (25 μunits/ml), while the response to larger doses of oxytocin were not affected. Similarly, pre-treatment of guinea-pig uteri with indomethacin completely abolished the response to low concentrations (5 μg/ml) of the apolar material. The response to high doses of apolar material (20 μg/ml) was also diminished by indomethacin.

In a similar manner the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA), at a concentration of 10 μmol/l abolished the contractile effects of the apolar fractions (20 μg/ml). The response to a higher concentration (50 μg/ml) of the apolar fraction was also dramatically decreased.

The effect on the oxytocic response of the apolar uterine stimulant in the presence of zinc (20 μmol/l) was investigated in 4 determinations. Relative to magnesium (Schild 1969), the oxytocic activity was potentiated 1.8-fold (± 0.4). In comparison, the potentiation of oxytocin was 2.7-fold (± 0.7).

The uterine stimulant activity of the apolar component was distinguished from oxytocin by the study of enzymic degradation (section 2.6.4). The amniotic fluid component was susceptible to the effects of chymotrypsin and carboxypeptidase, but resistant to the activity of trypsin and staphylococcal protease. In contrast, oxytocin was not susceptible to the effects of carboxypeptidase but was inactivated by the effects of trypsin, chymotrypsin and staphylococcal protease. Additionally, the activity of oxytocin was dramatically reduced by alkaline incubation while that of the apolar amniotic fluid component was unaffected (untreated 650 ± 58 units/mg, treated 740 ± 103 units/mg; n = 4).

The oxytocic response elicited by the apolar amniotic fluid

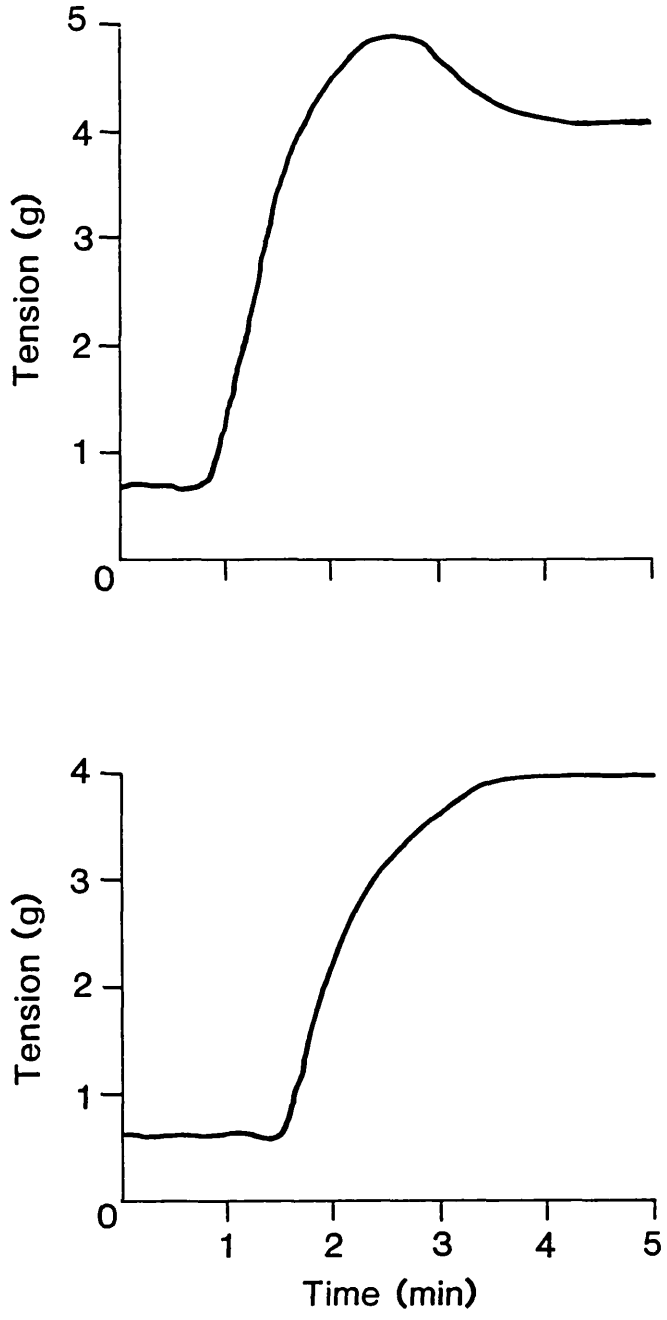


Fig. 33. A comparison of the stimulatory responses elicited by the low molecular weight, apolar oxytocic component (100 µg) of amniotic fluid and bradykinin (1 µg) on the isolated guinea-pig uterine horn.

The smooth muscle stimulant was added (t_0) and allowed to remain in contact with the preparation for 5 minutes, during which time the tension that developed in the tissue was monitored through a force displacement transducer. At the end of the contact time, the tissue was washed twice and allowed to recover for 10 minutes.

The response elicited by bradykinin is represented by the upper trace and that of amniotic fluid by the lower trace.

component was distinguished from bradykinin by the shape of the reaction curve (Fig. 33). Doses of the uterine stimulants were chosen which produced approximately equal responses at the end of the contact time (5 min). The latent period for contraction was shorter with bradykinin (0.6 ± 0.1 min) than that observed with the apolar amniotic fluid component (1.5 ± 0.2 min; $P < 0.005$, $n = 4$). In addition, the bradykinin-induced response revealed a maximum at approximately 2.6 min (± 0.4) and declined to the end of the contact period. In contrast, response elicited by the apolar uterine stimulant revealed a maximal value at approximately 3.8 min (± 0.4, $P < 0.01$) which was sustained to the end of the contact time.

Other oxytocic peptides, angiotensin II and substance P, were not compared with the polar or apolar low molecular weight components on the isolated uterine horn preparation. Together with oxytocin and bradykinin, they were distinguished from the amniotic fluid components by their elution position on reverse phase HPLC.

The oxytocic actions of gel filtration products was investigated on the uterine horn preparation ($n = 2$) and the human uterine strip ($n = 6$) with a limited number of samples. The mean activity of four samples of G10 material on rat uteri was 24 ± 7.5 units/mg. In contrast, the mean oxytocic activity of nine samples of gel filtration material on the human uterine strip preparation was 0.91 ± 0.31 units/mg.

8.3 Discussion

There is general agreement that amniotic fluid contains compounds that influence the activity of various smooth muscle preparations. The exact nature of these active principles is unknown. A situation analogous to that found with the antibacterial components of amniotic fluid, may apply. As discussed in chapter 1, the presence of many uterine stimulants in amniotic fluid has been reported. However, it has been more difficult to ascertain whether these components are present in pharmacologically-active concentrations. For example, although high concentrations of oxytocin have been reported, it has been shown that 90% of the oxytocin found in amniotic fluid is present in a biologically-inactive form (Seppala et al. 1972). Additionally, the presence of strong oxytocic activity in amniotic fluid does not imply a role in parturition.

Oxytocic activity was detected in all samples of lyophilised ultrafiltrates that were tested. Purification revealed that at least two oxytocic components were present, the greater proportion of activity residing in the more apolar fraction. The specific activity of the "apolar" fraction was much greater than that of the "polar" fraction. Neither factor was related to that with antibacterial activity.

The oxytocic activity of the polar and apolar components was not attributable to potassium ions, cholinergic stimulation (muscarinic or nicotinic), histamine or 5-hydroxytryptamine nor to the presence of prostaglandins. Additionally, the amniotic fluid components were distinct from bradykinin, angiotensin (II) and substance P. Pharmacologically, the apolar oxytocic component behaved in a manner very similar to that of oxytocin, particularly in its response to divalent cations (Schild 1969) and the action of the lipoxygenase inhibitor, nordihydroguaiaretic acid (Hall 1983).

Alternatively, the inhibitory action of FPL 55712 on the oxytocic action of the apolar component suggested a similarity to SRS-A (Aehringhaus et al. 1983).

The response of the apolar component was biochemically distinct from oxytocin. However, only peptides with a disulphide bridge are potentiated by divalent cations. Strongly active peptides are weakly potentiated while weakly active peptides are strongly potentiated (Schild 1969). Thus the apolar oxytocic component may be a powerfully-active cystine-containing peptide. The peptide nature of this fraction was further supported by the effect of proteases on the expression of oxytocic activity. Inactivation of the component by carboxypeptidase implied the presence of a peptide with an unrestricted carboxyl terminus; the resistance to attack of oxytocin is conferred by the amidated terminus. Additionally, the actions of chymotrypsin, trypsin and staphylococcal protease suggested the presence of aromatic amino acid residues but the absence of basic and glutamyl amino acids.

The three uterine tissues investigated, displayed a differential sensitivity to the oxytocic effects of the products of gel filtration. However, it could not be said that the sensitivity of the three tissues (rat, guinea-pig, human) to the gel filtration products varied as the ratio 24:6.5:0.91, as this assumed that the sensitivity of the tissues to oxytocin remained constant. The reverse argument may have been true. The results, however, did provide further evidence of the chemical dissimilarity of these smooth muscle stimulants.

The oxytocic activity of the gel filtration products of amniotic fluid obtained from patients of advanced maturity of pregnancy, was similar to that obtained from patients with diabetes,

hypertension or intra-uterine growth retardation. However, the oxytocic activity of three samples of amniotic fluid obtained from toxaemic patients was significantly greater. Interestingly, the pressor activity of whole amniotic fluid was greater in that obtained from toxaemic patients than that from normotensive subjects (Landesman et al. 1964).

Conclusions

In view of the importance of infection in obstetrics, with respect to both mother and baby, this study sought to examine the antibacterial activity of whole amniotic fluid.

The study was performed as follows:

1) The role of certain epidemiological factors in the expression of antibacterial activity of whole amniotic fluid.

2) The contribution of the low molecular weight antibacterial factor to the overall antibacterial effect was determined.

3) The influence of environmental factors on the expression of activity was investigated.

4) The low molecular weight antibacterial component was partially purified, a limited spectrum of antibacterial activity for the factor was examined and its presence in certain tissues of the uterine cavity investigated.

5) A tentative chemical structure was proposed for the low molecular weight compound and synthesis of the proposed molecule attempted. Its antibacterial activity was assessed but found to be much less active than expected. However, the exact structure of the synthetic peptide was not confirmed and this requires further study.

6) The oxytocic actions of the water-soluble fractions of amniotic fluid were demonstrated.not to be related to the low molecular weight antibacterial factor.

The initial studies revealed that whole amniotic fluid exerted a powerful antibacterial action against the indicator organism, *B. subtilis*. This effect was not related to maturity, parity or maternal age. Similarly, there was no relation with the indication for induction of labour or the source of amniotic fluid.

Environmental contaminants, however, did have profound effects on

the expression of activity: the activity of whole amniotic fluid was abolished by the presence of meconium but was obscured when staining with either blood or 'Hibitane' was evident. It is possible that meconium may act as a chelating agent to remove divalent cations that are essential for activity.

The antibacterial activity of samples of whole amniotic fluid was largely due to the presence of components with high molecular weights i.e. greater than 1000 daltons. Only one sample in four displayed low molecular weight antibacterial activity on initial testing, while the remainder displayed activity after concentration of the samples. The relevance to the in vivo situation, where the total amniotic fluid volume is changed over a 3 hour period, is difficult to assess. The presence or absence of low molecular weight activity bore a positive relation to the magnitude of the high molecular weight activity of the amniotic fluid samples. This has lead to the proposal that the low molecular weight antibacterial component is derived from the B-lysin molecule (Scane & Hawkins 1984).

Peptide extraction techniques permitted recovery of the low molecular weight antibacterial component which was determined to have a molecular weight near 450 daltons and was further purified by reverse phase high performance liquid chromatography. The purification factor achieved by this procedure, in weight terms, was approximately 30-fold. However, losses of bio-activity reduced this effect to a net purification of a mere 7-fold.

The high molecular weight antibacterial activity was thought to be largely due to the presence of the polypeptide B-lysin. Demonstration of the dependence of activity on the presence of divalent cations further supported the biochemical evidence of the

similarity of amniotic fluid and serum B-lysins. This also distinguished the high and low molecular weight antibacterial components: the high molecular weight factor being principally dependent on the divalent alkaline earth elements, magnesium and calcium, for activity while the low molecular weight component was dependent on the divalent transition metals, manganese, cobalt and zinc, for activity. These data further supported the peptide nature of the low molecular weight component as only certain amino acids, e.g. glutamate, tyrosine and proline, possess the ability to coordinate zinc.

The evidence for the peptide nature of the low molecular weight antibacterial component was supported by the results of the investigations of enzymology, affinity chromatography and thin layer chromatography. Analysis by fast atom bombardment mass spectroscopy suggested the amino acid composition Gln_2, Pro_1, Gly_1, or its isomer Glu_1, Gln_1, Pro_1, Gly_1, with an amidated carboxy-terminus (i.e. $-CO-NH_2$). A peptide with antibacterial activity was subsequently synthesised by the mixed anhydride method.

Antibacterial activity was only found in placental villous tissue and not in either amnionic or chorionic membranes. Low molecular weight antibacterial activity was present in all subcellular compartments of the placental tissue. Its similarity to the low molecular weight component of amniotic fluid was confirmed by the elution position on high performance liquid chromatography. However, although activity was detected in placental tissue, the site of production of this component was not identified.

The low molecular weight fraction of amniotic fluid also possessed both bronchodilator and oxytocic activity. Reverse phase HPLC was used to distinguish the components with these actions from

that with antibacterial activity. The bronchodilator activity of the lyophilised ultrafiltrates was thought to be due to ionic effects, while the oxytocic action was resolved into two fractions. The apolar oxytocic component accounted for the greater proportion of activity and displayed a pharmacological profile similar to, but different from, oxytocin.

By convention, peptides of a molecular weight up to 10,000 daltons are known as polypeptides and above that as proteins. Biologically-active polypeptides are common and range in size from a few residues (e.g. muramyl dipeptide and contraceptive tetrapeptide) upwards. Several peptides with antibacterial activity are available commercially; Bacitracin A (a substituted decapeptide), Gramicidin (11 residues), Subtilin (32 residues) and Viomycin (a substituted pentapeptide) while some antibiotics possess a peptide component to their structure e.g. Polymyxins. Many other antibacterial peptides have been isolated from algal, bacterial and fungal sources (e.g. Hypelcin A, Fujita et al. 1979; A-10947, Yaginuma et al. 1979). Cationic antibacterial peptides have also been reported in the rabbit (Carrol & Martinez 1981a,b),guinea-pig (Gleich et al. 1974) and human (Zeya & Spitznagel 1968, Gleich et al. 1976). Further, specific proteins have been detected in the plasma of pregnant women, which are not found in non pregnant female subjects or in males (Lin et al. 1974).

A common feature of many small bioactive peptides is the presence of the amino acid proline. It is present, for example, in tuftsin (a chemotactic tetrapeptide, Najjar 1974), a contraceptive tetrapeptide (Kent 1975), and a cell attachment promoting peptide (Pierschbacher & Ruoslahti 1984). The presence of this amino acid confers resistance to enzyme attack by trypsin, chymotrypsin and

glutamyl protease (Desnuelle 1960, Smyth 1967, Houmard & Drapean 1972). It may also act as a zinc binding ligand. This latter property is also confered by the presence of another amino acid, glutamine (Bere & Helene 1979).

The investigation of antibacterial activity within the placenta revealed two low molecular weight forms with antibacterial activity. Interestingly, the synthetic peptide displayed low antibacterial activity in relation to that of the native peptide. A similar situation applied each case: two factors with antibacterial activity, the least active of which also displayed a shallower growth inhibition curve.

The detection of antibacterial activity in sub-cellular compartments of the placenta, like its detection in amniotic fluid, does not necessarily indicate the site of production. What has been demonstrated is that, at delivery, the placental tissues are capable of exerting an antibacterial effect. Also, the highest concentration of the low molecular weight antibacterial component is found in that sub-cellular compartment associated with extra-cellular secretion. Further work could examine the site(s) of synthesis of the low molecular weight antibacterial peptide.

Several peptides which stimulate smooth muscle are well documented (e.g. angiotensin, bradykinin, kallidin, neurotensin, oxytocin, and vasopressin). Many of these peptides have been detected in amniotic fluid. They were distinct from the low molecular weight oxytocic peptide detected in this study. Hunter & Howard (1960) also reported the presence of an oxytocic peptide, hysterotonin, in amniotic fluid, but were only able to detect it in amniotic fluid from toxaemic patients. The results of this study indicated low levels of oxytocic activity in amniotic fluid which

were raised in samples obtained from toxaemic patients. It is possible that these results simply reflect a more sensitive assay system than that used by Hunter & Howard (1960).

This study had many objectives. The approach to assay of antibacterial activity by use of the agar plate diffusion technique demonstrated that the antibacterial activity of amniotic fluid could be determined reliably, simply, reproducibly and accurately. Having established a viable technique for assay, it thus became possible to establish the relative importance of the contribution of the low molecular weight antibacterial component to the overall antibacterial effect of amniotic fluid.

Examination of the antibacterial activity of the low molecular weight amniotic fluid component against a limited spectrum of bacteria revealed that an apparently similar level of inhibition was exerted against both Gram-positive and Gram-negative micro-organisms. Since the principal antibacterial factor in amniotic fluid, B-lysin, is chiefly active against Gram-positive bacteria (Schlievert et al. 1977), the role of the low molecular weight component may be to provide additional antibacterial cover against Gram-negative organisms. It would have been interesting to perform the comparison of the relative magnitudes of antibacterial activity of high molecular weight and low molecular weight amniotic fluid fractions, as shown in chapter 4, but using a Gram-negative micro-organism.

The principal objective of the study was to establish the identity of the low molecular weight component with antibacterial activity. Absolute identification was thwarted by the need to develop techniques which minimised the loss of bioactivity on purification. Partial identification was achieved, resulting in the synthesis of a peptide with antibacterial activity together with the demonstration

of its dependence on the presence of zinc for activity against B. subtilis.

Overall, the powerful antibacterial action exerted by the low molecular weight peptide bears further examination. Particularly, in view of the fact that the synthetic peptide exerted a far weaker action than had been anticipated. Future work on the antibacterial activity of the low molecular weight peptide should first seek methods which permit purification with minimal loss of activity. Use of alternative stationary phases in HPLC separations, as suggested in chapter 4, may offer the best prospect for further purification studies. Work to determine the exact chemical nature, particularly the interaction with zinc, together with a complete pharmacological screen would be required prior to the use of this peptide as an antibiotic. However, in view of the toxicity of systemically administered polypeptide antibiotics, it is possible that the low molecular weight antibacterial peptide might be of greater use for topical applications.

The oxytocic actions of the low molecular weight fraction of amniotic fluid also bears further examination. The precise chemical nature of the apolar uterine stimulant particularly requires investigation. Its action on other smooth muscle preparations should be determined.

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