THE MICROBIOLOGY OF BOVINE SEMEN
AND
THE ANTIMICROBIAL ACTIVITY OF BOVINE SEMINAL PLASMA

A thesis presented in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy in Microbiology at
Massey University.

Linda May Schollum

December, 1977.

ABSTRACT

Microbial populations in semen from 18 bulls used for artificial breeding, varied between 30 and 560,000 organisms/cm^3, with a mean of 3,300/cm^3. Wide day to day fluctuations occurred in the microbial content of semen and there was no correlation between the total microbial counts, the proportion of these organisms sensitive to bovine seminal plasma inhibition and the measureable amount of antibacterial activity in a given semen sample. All semen samples had antibacterial activity, the amount being relatively constant for a given animal; marked differences occurred between breeds. Sixty-eight percent of the bacteria isolated from semen (representing ten genera) were inhibited by seminal plasma. Together with other data, this supports the view that most semen micro-organisms are transient contaminants from outside the reproductive tract, derived mainly from the preputial cavity.

The most common organisms in bovine semen were species of Bacillus, Corynebacterium and Micrococcus, but twelve other bacterial genera and two yeast species were also represented among the isolates. Most semen microorganisms were sensitive to several antibiotics, with pseudomonads, coliforms and yeasts proving the most resistant. The control of these organisms is important since they may grow in extended semen and have been implicated in bovine genital disorders. Chloramphenicol and streptomycin are routinely used in semen extenders and have been highly effective in controlling bacterial growth; penicillin has been less effective.

At least three antimicrobial activities have been observed in bovine seminal plasma: (i) an antiviral activity, (ii) a heat labile (60^{o}C for 60 minutes) antibacterial activity and (iii) a heat stable antibacterial activity. Research has centered on the heat stable fraction which is sensitive to proteolytic enzymes but not to periodate treatment. This heat stable fraction can be purified by adsorption onto highly polymerised deoxyribonucleic acid (DNA). It does not resemble lysozyme, polylysine, salmine or spermidine in antimicrobial spectrum, electrophoretic properties or amino acid composition. Data from dialysis, polyacrylamide gel electrophoresis, ultrafiltration and ultracentrifugation indicate that the active moiety occurs in varying molecular weight aggregates, caused by self association and interaction with other microsolutes present in its environment. At least two of the aggregates (cationic at pH8.6) are anti-bacterial by the overlay technique which detects biological activity in electrophoresed proteins. However, it migrates as a single band (molecular

weight 25,000) in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, and has a single N terminal amino acid. When compared with antibacterial compounds described by other workers, the antimicrobial proteins from bovine seminal plasma were found to have a unique chemical composition.

Bovine seminal plasma proteins had a rapid lethal effect on both Gram-positive and Gram-negative bacteria as well as ureaplasmas and mycoplasmas. Bacterial resistance to bovine seminal plasma exhibited a degree of correlation with resistance to antibiotics affecting cell membrane integrity. Detection of cytoplasmic leakage, loss of accumulated labelled compounds and failure to accumulate a labelled amino acid by bacteria in contact with seminal plasma supports the hypothesis that membrane damage is induced by bovine seminal plasma antimicrobial proteins. Resistance of Pseudomonas aeruginosa to bovine seminal plasma appears to be due to the bacterial cell wall preventing access of the active moiety to the cell membrane.

Bovine seminal plasma antimicrobial cationic proteins may well contribute to an efficient primary nonspecific defence mechanism of the genital tissues. Few microorganisms colonise the upper reproductive tract; ascending infections from the lower urethra and preputial cavity, where bacterial populations are large, are uncommon. Only bacteria shown to be resistant to antimicrobial factors present in bovine seminal plasma have been implicated in bovine male reproductive tract infections. During coitus the antimicrobial activity may also protect the female genital tract from infection.

ACKNOWLEDGEMENTS

I would like to personally thank the following:

Professor D.F. Bacon and Dr B.D.W. Jarvis, for their supervision and guidance.

Mr P. Shannon and Dr K. MacMillan, of the New Zealand Dairy Board Artificial Breeding Service, for the provision of semen and seminal plasma, and for their encouragement and friendship.

Dr J. Clarke, for his work on the antiviral properties of bovine seminal plasma, and for his interest throughout the thesis.

Dr G. Midwinter, for the amino acid analysis of material isolated from bovine seminal plasma.

Dr C. Moore, for assistance with ultracentrifugation techniques.

Professor N.P. Markham, Otago University, for the provision of Ureaplasma strains. The Veterinary Pathology Department, Massey University, for Leptospira pomona and L. hardjo cultures, and the Biochemistry Section for the loan of electrophoresis equipment.

My parents, for their assistance with photographs and figures, and for their never-ending support.

Thanks also to all my other colleagues, family and friends, too numerous to name, but who helped and encouraged me throughout this thesis.

TABLE of CONTENTS.

/ . . .

TABLE of CONTENTS (contd)

/ . . .

TABLE of CONTENTS (contd)

/ . . .

TABLE of CONTENTS (contd)

/ . . .

TABLE of CONTENTS (contd)

/ . . .

TABLE of CONTENTS (contd)

LIST of TABLES

/ . . .

LIST of TABLES (contd)

/ . . .

LIST of TABLES (contd)

LIST of FIGURES

/ . . .

LIST of FIGURES (contd)

/ . . .

LIST of FIGURES (contd)

/ . . .

LIST of FIGURES (contd)

LIST of PLATES

INTRODUCTION

Semen microbiology as well as reproductive physiology, has become a topic of increasing importance with the growing interest in improving breeding efficiencies, correcting reproductive disorders, and enhancing the production potentials of artificial breeding. Microbiological aspects which may be associated with low breeding efficiencies or sterility are important in both natural reproduction and in artificial breeding practice. In addition, the use of a single ejaculate to inseminate a large number of cows provides a technique which, without correct safeguards, could lead to widespread dissemination of a specific pathogen throughout several herds in different farming regions. For this reason, periodic microbiological monitoring of semen used for artificial breeding, and investigations of the mechanisms preventing reproductive tract diseases in bulls and their transmission to serviced cows, are of considerable importance.

CHAPTER 1: THE MICROBIOLOGY OF BOVINE SEMEN

1.1 Microbial Populations in Bovine Semen

The total microbial population and the species characteristic of the natural flora of bovine semen have been found to vary considerably. In part, this may be due to the manner in which the semen was obtained and the treatment of the animals before collection. Although various methods have been used to obtain semen for microbiological studies, the use of an artificial vagina and normal artificial breeding techniques seems to be the method of preference.

An artificial vagina consists of a long rubber jacket which extends over a test-tube in which the ejaculate is collected; the rubber jacket is sterilised by flushing with alcohol and the collection test-tube by heat sterilisation. When the bull is stimulated and mounts a teaser animal, the rubber sheath of the artificial vagina is slipped over the penis and held in position until ejaculation has occurred. The test-tube is then removed from the rubber sheath and stoppered. This method avoids the possibility of contamination of the semen by vaginal flora which may occur, in spite of preliminary douching and disinfection, if the semen is recovered from the vagina of cows immediately after service (Webster 1932). Electro-ejaculation, which uses a light electrical charge to stimulate ejaculation into a collection vessel, has also been used for semen collection. It often yields ejaculates which are different in composition and greater in volume than those collected by normal ejaculation (Mann 1964). Semen collected in such a manner is therefore not representative of normal bovine genital tract secretions. Normal ejaculation into an artificial vagina provides samples representative of natural semen in both chemical and microbiological characteristics.

Gilman (1921) investigated the genital tracts of bulls shortly after slaughter and concluded that normal healthy bulls had reproductive tracts either free of or with very low numbers of bacteria. Ruebke (1951) could isolate no microorganisms from testes, epididymis, ampullae of vas deferentia, prostate or bulbo-urethral glands of healthy animals after slaughter. The urethra, caudal to the prostate through to the external urethral orifice, the prepuce and the glans penis were, however, found to contain viable microorganisms. Hatziolos (1937) attempted to obtain semen as free of bacterial contamination as possible, but found that no ejaculate was entirely free of bacteria. Thus although fresh semen from healthy bulls is theoretically very low in bacterial numbers, semen varies considerably in its bacteriological quality.

Of all sources outside the reproductive tissues, the preputial cavity is considered the main source of semen contaminating microorganisms. Salisbury et al (1939) found populations of 3.0×10^5 to 3.0×10^7 microorganisms/cm^3 of semen collected by artificial vagina with no special preparation of the bull. These numbers were reduced to 1.0×10^4 to 1.0×10^5 organisms/cm^3 by thorough washing of the underline and douching of the sheath. A similar decrease in the microbial population of semen after washing the prepuce prior to semen collection was also reported by Hashimoto (1963). Gunsalus et al (1941) reported total viable counts with a logarithmic average of 2.25×10^5 organisms/cm^3 and a range of 1.0×10^3 to 2.2×10^7 in semen from bulls with muddy underlines and sheaths. Semen from bulls which were clean about underline and sheath had microbial populations ranging from 1.0×10^3 to 4.3×10^6, with a logarithmic average of 2.2×10^4 organisms/cm^3. They also found that careful washing of the underline, clipping of the hair about the prepuce and flushing of the sheath with sterile distilled water, reduced by 10 to 100 fold the number of microorganisms in the semen of a previously dirty bull. Meredith (1970) found that semen from bulls that everted the preputial epithelium and therefore increased the likelihood of contamination from the external environment had greater microbial populations (average 3.7×10^5 organisms/cm^3) than normal animals (average 4.0×10^4). Reddy et al (1971) further studied the contribution of the flora of the prepuce to semen contamination and reported that the semen of bulls with tucked up prepuces had lower average microbial counts than those with pendulous prepuces. As in many studies of the total viable counts in semen, however, the difference was not statistically significant due to the wide variations in the microbial populations within each group. The fact that most of the bacterial species present in the prepuce were also present in semen does emphasise the importance of the prepuce as a source of bacterial contamination in semen. These workers also confirmed the earlier reports that flushing the prepuce before semen was collected markedly lowered the microbial content of the semen. They found that the total viable counts of semen from these bulls remained below the prewash levels for approximately 45 days after washing. Zemjanis (1962), therefore, recommends flushing the preputial cavity prior to semen collection to reduce the microbial content of semen in all routine collections.

It can be concluded that the handling of the animals just before semen collection is important in determining the microbial flora of their semen. Some of the differences between reports on the microbial flora of semen may also be associated with differences related to the general management of individual herds or to management practices associated with different

geographical areas. Singleton & Simmons (1969) investigated the semen of bulls housed under intensive conditions and found a high incidence of coliforms in their semen. Previously, coliforms were claimed to occur only occasionally in bovine semen (Prince et al 1949) and their presence was attributed to faecal contamination. Under a stall system of management, faecal contamination of the prepuce could reach a much higher level than would be expected under less intensive conditions; this expectation was supported by the persistence of coliforms in the semen of bulls housed under intensive conditions. The importance of general animal management on semen microbiology was also indicated in the studies by Gunsalus et al (1941), discussed above, in which bulls maintained under muddy conditions produced semen with higher microbial numbers than bulls kept in clean surroundings.

Even within a single herd, there are large variations in the microbial populations of semen from different bulls and in ejaculates collected at different times from the same bull. The greatest variations were reported by Almquist et al (1949b) with a range of 5.0×10^1 to 3.3×10^6 organisms/cm^3 and by Gunsalus et al (1941) with a range of 1.0×10^3 to 2.2×10^7 organisms/cm^3 of semen. Other investigators have reported microbial populations in bovine semen within these ranges (Salisbury et al 1939; Almquist et al 1949a; Foote & Bratton 1950b; Hashimoto 1963; Meredith 1970; Reddy et al 1971).

There is some indication that young bulls may produce semen with lower microbial populations than older animals. Almquist et al (1949b) reported that there was an increase in the microbial populations of semen collected from bulls of increasing age, although this difference was found statistically to be non-significant. Reddy et al (1971), however, observed that young bulls had significantly lower total counts than mature and old animals, although there was no significant difference between the latter two age groups.

In addition, when two ejaculates are collected within a few minutes of each other, the second often contains fewer microorganisms than the first (Salisbury et al 1939; Almquist et al 1949b; Sorel 1961). For example, Almquist et al (1949b) found that in 21 cases of the 32 examined i.e. 65%, the first ejaculate contained higher microbial numbers than the second ejaculate. However, the average total count reported for each ejaculate (2.2×10^5 organisms/cm^3 and 1.3×10^5 organisms/cm^3 respectively) are misleading since arithmetic rather than logarithmic averages were calculated, and the difference is not sufficiently large to be considered significant. Such trends in the microbial population of semen have been

reported in various studies (see above), but too often statistical analysis has shown them to be non significant due to the considerable variation in total colony counts within each group analysed.

1.2 The Importance of Total Microbial Numbers in Bovine Semen

There has been considerable discussion about the effect of micro-organisms on the fertility of a sire and on the survival of spermatozoa in semen collected and stored for artificial breeding.

Early workers believed that there was no direct evidence that bacteria had any detrimental effect on breeding efficiencies (Salisbury et al 1939), although they questioned the advisability of inseminating a cow with semen containing large numbers of pus-formers such as the pseudomonads. For this reason, combinations of antibiotics including penicillin, streptomycin, polymyxin and sulphonamides, were tested and found to control bacterial growth in semen while preserving sperm liveability (Foote & Salisbury 1948; Almquist et al 1949a; Foote and Bratton 1950b). There have, however, been conflicting reports on the effect of antibiotic addition on the conception rates of semen used in artificial breeding. Holt (1952) reported that sulphanilamide had no effect on the conception rate. In this study no controls lacking the antibiotics were included. In contrast, a significant increase in the fertility of semen diluted with an extender containing sulphanilamide was noted by Salisbury & Knodt (1947) and Salisbury & Bratton (1948). Because the response of all samples was so uniform, they considered it to be a metabolic effect rather than one based solely on the control of the microbial population in the semen. Foote & Bratton (1950a) reported that the addition of penicillin, streptomycin or a combination of these plus polymyxin and sulphanilamide increased the overall fertility level of bovine semen used for artificial breeding; the greatest increase occurred in low fertility bulls. Similarly, Easterbrooks et al (1950) found that the addition of streptomycin to diluted bovine semen increased the conception rates of even high fertility bulls. Many investigators, however, have reported that the control of bacterial growth in semen by the addition of various antibiotics to semen extenders has had no significant effect on the conception rates of bulls with relatively high breeding efficiencies (Almquist et al 1946; Mixner 1949; Almquist & Prince 1950). But it is generally agreed that significant improvements do occur in the fertility of semen from some relatively infertile animals (Almquist 1948; Almquist 1949a; Almquist 1949b; Almquist & Prince 1950;

Almquist 1951; Willett & Ohms 1955).

As well as the increase in the levels of conception in semen from low fertility animals, the addition of antibiotics to semen reduced the number of cows returning to heat after abnormally long intervals. Such returns reflect early embryonic or foetal deaths and/or pyelometritis. Indirect evidence is present in the work of Salisbury & Bratton (1948); Easterbrooks et al (1950) and Foote & Bratton (1952), indicating that antibiotic addition to semen effectively reduces embryonic mortality by controlling infectious agents present in semen. Decreases in embryonic deaths on the addition of antibiotics to semen, especially from bulls of generally low fertility have been confirmed by Olds et al (1951); Erb & Flerchinger (1954) and Willett & Ohms (1955).

Gilman (1921) isolated large numbers of bacteria from the genital tracts of sterile bulls and bulls with impaired fertility, whereas semen from fertile bulls contained few if any bacteria. However, no relationship could be established between the number of microorganisms present in semen and, either the length of time, based on sperm motility criteria, that semen could be stored (Edmondson et al 1948) or the general fertility level of bulls (Almquist et al 1949b). It is therefore likely that the nature of the microbial flora of bovine semen rather than the total population of microorganisms, determines the fertilising capacity of a particular sample of semen. The enhancement of conception rates and the lowering of embryonic mortality rates by the addition of antibiotics to semen from relatively low fertility bulls, indicates that low breeding efficiencies in bulls may be associated with the presence of certain specific bacteria in or near the reproductive tract.

1.3 The Composition of the Microbial Flora of Bovine Semen

The types of microorganisms present in semen samples are generally considered to be of greater importance than the total microbial numbers. The microbial flora of semen from healthy bulls is essentially similar in most reported studies, although the relative frequency of a particular organism may vary between bull populations. For example, Singleton & Simmons (1969) found a higher frequency of coliforms in bulls housed in stalls than many other investigators studying free ranging animals. These increased coliform counts are probably due to greater faecal contamination of the prepuce in bulls housed under intensive conditions.

The bacteria most commonly isolated from the semen of healthy bulls

are:

(a) COLIFORMS (Gilman 1921; Hatziolos 1937; Gunsalus et al 1941; Edmondson et al 1948; Prince et al 1949; Ruebke 1951; Sorel 1961; Hashimoto 1963; De & Chowdhury 1965; Blom & Dam 1967; Singleton & Simmons 1969; Meredith 1970; Reddy et al 1971).

(b) CORYNEBACTERIA and DIPHTHEROIDS (Webster 1932; Salisbury et al 1939; Gunsalus et al 1941; Prince et al 1949; Ruebke 1951; Sorel 1961; Hashimoto 1963; Kazda 1963; De & Chowdhury 1965; Singleton & Simmons 1969; Meredith 1970).

(c) MICROCOCCI (Gilman 1921; Webster 1932; Hatziolos 1937; Edmondson et al 1948; Prince et al 1949; Ruebke 1951; Sorel 1961; Hashimoto 1963; Singleton & Simmons 1969).

(d) BACILLUS SPP (Hatziolos 1937; Gunsalus et al 1941; Edmondson et al 1948; Ruebke 1951; Sorel 1961; Hashimoto 1963; De & Chowdhury 1965; Singleton & Simmons 1969; Reddy et al 1971).

(e) PROTEUS SPP (Hatziolos 1937; Ruebke 1951; Sorel 1961; Hashimoto 1963; De & Chowdhury 1965; Singleton & Simmons 1969; Meredith 1970; Reddy et al 1971).

Additional organisms have been isolated at lower frequencies. They are probably not part of the normal microflora of healthy semen but occur as transient contaminants of the prepuce from bedding, manure, soil and air. Their numbers can often be substantially decreased by cleaning the bull before semen collection. These species include:

(f) PSEUDOMONADS (Gilman 1921; Hatziolos 1937; Edmondson et al 1948; Prince et al 1949; Ruebke 1951; Hashimoto 1963; Kazda 1963; De & Chowdhury 1965; Singleton & Simmons 1969; Meredith 1970; Reddy et al 1971).

(g) STAPHYLOCOCCI (Salisbury et al 1939; Gunsalus et al 1941; Edmondson et al 1948; Hashimoto 1963; De & Chowdhury 1965; Singleton & Simmons 1969; Meredith 1970; Reddy et al 1971).

(h) STREPTOCOCCI (reported to occur in bovine semen by Gilman 1921 ; Edmondson et al 1948 ; Ruebke 1951 ; Hashimoto 1963 ; De & Chowdhury 1965 ; Singleton & Simmons 1969 ; and Meredith 1970 ; reported as absent from bovine semen by Webster 1932 ; Gunsalus et al 1941 ; and Prince et al 1949).

(i) ALCALIGENES (Prince et al 1949; Ruebke 1951; Hashimoto 1963; Reddy et al 1971).

(j) ACTINOMYCES (Edmondson et al 1948).

(k) AEROBACTER (Ruebke 1951).

(l) BREVIBACTERIUM (Bacterium ammoniagenes) (Ruebke 1951).

(m) CHROMOBACTERIUM (Meredith 1970).

(n) ENTEROCOCCI (Sorel 1961).

(o) FLAVOBACTERIUM (Prince et al 1949).

(p) GAFFKYA (Hashimoto 1963).

(q) KLEBSIELLA (Singleton & Simmons 1969).

(r) PEPTIDOCOCCUS (Micrococcus anaerobis) (Ruebke 1951).

(s) PROVIDENTIA (Hashimoto 1963).

(t) SERRATIA (Singleton & Simmons 1969).

(u) YEAST (Edmondson et al 1948).

Bovine semen also contains variable populations of fungi. Rob & Toman (1971) isolated mycelial fungi from 23% of semen samples and 22 - 36% of preputial washings from breeding bulls in Czeckoslovakia. The predominant genera were Penicillium, Cladosporium, Aspergillus, Alternaria and Thamnidium. Richard et al (1976) also isolated several non-yeast fungi from bovine semen but did not attempt to identify the organisms involved. They did, however, identify nine species of mycelial yeasts, the most commonly isolated being Candida parapsilosis and C. tropicalis. In addition strains of C. sake, C. curvata, C. krusei, C. lambrica, Hansenula anomala and Torulopsis candida were also cultured from bovine semen.

Mycoplasma species (some unidentified, many classified as M. bovigenitalium some, as Acholeplasma laidlawii, and a few as M. agalactiae) have been cultured from the prepuce and semen of bulls exhibiting no evidence of pathological conditions. They have been isolated from 66% of penial-preputial swabbings and 24% of raw semen from Canadian bulls (Onoviran et al 1975), although Langford (1974) reported a lower (14%) incidence. 27% of preputial washings and 7 - 8% of semen samples from Danish breeding bulls (Ern et al 1967; Blom & Erno 1970), 27% of semen samples from Polish bulls (Zgorniak-Nowosielska & Branny 1970; Branny & Zgorniak-Nowosielska 1971) and 83% of preputial mucosa samples in U.S.A.

(Jasper et al 1974) were found to contain Mycoplasmas.

T Mycoplasmas (Ureaplasmas) have been isolated from the reproductive tract of cattle in the U.S.A. (Livingston & Gauer 1974), 35% of penial-preputial swabbings and 24% of raw semen from Canadian bulls (Onoviran et al 1975), and from either preputial cavity or semen of 82% of British bulls examined (Taylor-Robinson et al 1969). A low incidence was again reported by Langford (1974) with 13% incidence of ureaplasmas in preputial secretions and semen of Canadian animals.

1.4 The Significance of Certain Microorganisms in Bovine Semen, their Role in Infertility and the Likelihood of their Transmission to Serviced Cows

In cases of specific bovine reproductive tract diseases, the causitive organisms may be ejaculated in the semen, which could then serve as a source of infection for cows serviced with the contaminated semen. For example, Brucella abortus has been isolated from cases of seminal vesiculitis (Lagerlof 1936) and from the seminal vesicles of a bull with a history of sterility (Gilman & Hopper 1925). It has also been associated with other bovine genital tract diseases (ampullitis, orchitis, epididymitis) affecting the fertility of the infected animals (reviewed by Bagshaw & Ladds 1974a, and Humphrey & Ladds 1975). Campylobacter (Vibrio) fetus can be excreted in bovine semen (Dunn et al 1965; Winter et al 1965), is present in preputial washings (Clark et al 1974a), and is able to survive in deep frozen semen (Adler 1966). Vibriosis has been detected in 50% of bulls in Britain (Philpott 1969), 30% of bulls in Canada (Ruckerbauer et al 1974) and 37% of animals in the United States (Winter et al 1970). Danish bulls are now free of Vibrio fetus infection following an intensive eradication scheme, involving repeated test matings and mass treatment with streptomycin (Adler 1965). Leptospira pomona was transmitted to serviced cows by infected bulls during coitus or by diluted semen from these animals when this was used for intravaginal inoculation in artificial breeding (Sleight 1965). Other Leptospira species may also be associated with genital infection and venereal transmission of the disease to serviced cows (Amatredjo & Campbell 1975). Mycobacterium tuberculosis has been found in the semen of bulls with seminal vesiculitis (Lagerlof 1936), and Mycobacterium paratuberculosis (M. johnei) has been isolated from the testes, bladder and semen of clinically healthy animals (Lukashev et al 1963). It has also been found in the faeces, genital organs and semen of infected bulls (Tunkl & Aleraj 1965; Larsen & Kopecky 1970). It is therefore possible that transmission to serviced cows via contaminated semen does

occur when semen is collected from infected animals. Trichomonas fetus is present in semen and preputial washings of infected animals (Joyner & Miller 1952; Zemjanis 1962; Sunaikin 1971), and is able to survive in semen held at 5'C for up to nine days (Fitzgerald et al 1954).

Thus, artificial breeding may be important in the transmission of specific diseases, e.g. brucellosis, leptospirosis, tuberculosis (Johnei's disease), trichomoniasis and vibriosis, to large numbers of serviced cows. For this reason all bulls for use in artificial breeding practices are checked serologically for these infections.

In addition to these monitored diseases, however, certain other microorganisms also affect bull fertility and may infect the female genital tract following insemination with contaminated semen. Bulls with seminal vesiculitis produced post-coital vaginal discharge in cows mated with these animals (Galloway 1964), and service by a low fertility bull has been found to lower the fertility of repeat service cows even though the repeat services were with semen from bulls of high fertility (Flerchinger & Erb 1953). This was possibly due to an infection in the reproductive tract of the cow resulting from insemination with semen from a low fertility bull containing a large microbial population.

Several genera of bacteria have been reported to be responsible for reproductive disorders in bulls and cows serviced by these animals. Two recent reviews, Bagshaw & Ladds (1974a) and Humphrey & Ladds (1975) discuss the pathology of the accessory sex glands of the bull, and of bovine testes and epididymis respectively. The organisms involved in pathological conditions of the bovine reproductive tract are discussed below.

Alpha haemolytic streptococci were found in large numbers in cultures of semen from bulls in herds with endemic temporary sterility. This organism was recovered from the testes, epididymis and seminal vesicles of these bulls at the time of slaughter, although they were not found in cultures from healthy bulls or from bulls of low fertility characterised by oligospermia or highly abnormal sperm. The cows serviced by these bulls also exhibited enzootic sterility together with evidence of cervicitis, and frequently yielded similar alpha haemolytic streptococci on culture (Webster 1932). Streptococci have also been isolated from cases of seminal vesiculitis (Lagerlof 1936; Galloway 1964). Sorel (1961) reported that Streptococcus pyogenes, alone or in combination with Escherichia coli or Pseudomonas aeruginosa, occurred in chronic infections of bulls with low fertility. These organisms have also been associated with ovaritis,

metritis, sterility and abortion in cows (Prince et al 1949).

Staphylococcus aureus and Staphylococcus albus[1] have been isolated in large numbers from cases of cervitis (Webster 1932) and Staphylococcus pyogenes[1] occurred in the semen of certain bulls with low fertility (Sorel 1961). Pounden et al (1947) isolated S. albus[1] from the semen of a bull which appeared to be identical to that obtained in large numbers from the pus and exudate surrounding aborted feti from cows previously inseminated with semen collected from the same bull.

Some Pseudomonas species isolated from bovine semen are saprophytes from external contamination; others are definite pathogens and were frequently associated with Staphylococcus pyogenes[1] in the semen of bulls with low fertility. Semen from these bulls contained high numbers of dead and abnormal sperm (Sorel 1961). The persistent presence of Ps. aeruginosa in the semen of infertile bulls also indicated that it may be associated with individual cases of infertility involving low sperm motility, a high percentage of morphologically abnormal sperm (Prince et al 1949) and low breeding efficiencies (Gunsalus et al 1944). Pseudomonas species have been isolated from bulls with balano-posthitis, and is known to be spermicidal (Lusis & Soltys 1971). Ps pyocyanea[2] was found in the semen of five bulls (Gunsalus et al 1941). Of these five animals, three were culled from the artificial breeding herd, another became sterile and the last produced semen of low fertility. Semen contaminated with Ps. aeruginosa produced uteritis, cervicitis, vaginitis and metritis in heifers serviced with this semen (Getty & Eilis 1967).

Richardson & Smith (1968) could find no evidence for the pathogenicity of Corynebacterium haemolyticum, but Corynebacterium renale has been implicated in bovine seminal vesiculitis (Galloway 1964), and a diphtheroid was found to be responsible for ulcerative vulvitis and posthitis (Dent 1971). In addition, Corynebacterium pyogenes has been isolated from abcesses and lesions in posthitis and prolapse of the prepuce in cattle (Donaldson & Aubrey 1960), and from lesions in the seminal vesicles of low fertility bulls (Sorel 1961), or in cases of seminal vesiculitis (Lagerlof 1936; Galloway 1964; Bagshaw & Ladds 1974b).

Van Tonder & Bolton (1970) isolated Actinobacillus seminis from a bull with bilaterial epididymitis, and also believed it to be responsible for two cases of orchitis, on serological evidence. Actinobacillus

FOOTNOTES: 1 currently classified as Staphylococcus aureus (Bergey 1974)

2 currently classified as Pseudomonas aeruginosa (Bergey 1974)

actinoides has been associated with bovine seminal vesiculitis (Jones et al 1964).

Proteus and Escherichia spp. appear to be transition organisms between the clearly saprophytic and the pathogenic microorganisms. They may be present in semen with reduced fertility and increased sperm abnormalities, but they appear to reflect a general lowering of the animals health, rather than being the causal agents of these symptoms (Sorel 1961). Escherichia coli, however, has been reported in the inflamed seminal vesicles of sterile or near sterile bulls (Kazda 1963; Galloway 1964), and has been associated with a variety of reproductive disorders in cows (Prince et al 1949).

Other bacterial species occasionally involved in bovine reproductive tract disorders are Actinomyces bovis (Kimball et al 1954), Haemophilus (Humphrey & Ladds 1975), Nocardia farcinica and other Nocardia spp (Awad 1960; Galloway 1964), and Salmonella (Humphrey & Ladds 1975).

These bacterial genera can therefore be considered responsible for reproductive tract infections of the bull and for lowered fertilising capabilities of its semen. This decrease in fertility may be associated with greater sperm abnormalities, or with the transmission of infection to serviced cows leading to a variety of disorders of the female reproductive tract including sterility or lowered conception rates due to ovaritis and metritis and to increased embryonic deaths associated with such genital infections.

In addition to the potential pathogens present in bovine semen affecting fertility, certain non-pathogenic bacteria may lower the fertility of semen for use in artificial breeding by competing with the sperm for nutrients or by producing toxins and metabolic end products which may be harmful to sperm. A definite correlation exists between high numbers of haemolytic bacteria in a semen sample and a reduced storage capacity for such a sample (Edmondson et al 1948). In addition, Clostridium welchii, Escherichia freundii[1], Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa and Streptococcus viridans have been shown to have marked spermicidal effects when present in semen (Matthews & Buxton 1951; Kazda 1963; Lusis & Soltys 1971).

In many chronic reproductive diseases e.g. seminal vesiculitis, bacteriological investigations have proved negative. Such inflammatory conditions may be associated with mycoplasmal, chlamydial or viral infections of the bovine reproductive tract.

FOOTNOTE: Currently classified as Citrobacter freundii (Bergey 1974).

Mycoplasma species have been associated with low fertility, low conception rates, vulvovaginitis, endometritis, salpingitis, mastitis and abortion (Jasper 1967), although they have also been isolated from the genital tract and semen of animals with no clinical symptoms (Ern et al 1967; Blom & Erno 1970; Zgorniak-Nowosielska & Branny 1970; Branny & Zgorniak-Nowosielska 1971). Mycoplasma bovigenitalium has been isolated from the semen and reproductive organs of animals with chronic seminal vesiculitis (Blom & Erno 1967), and with orchitis and epididymitis (Al-Aubaidi et al 1972). Mycoplasma bovigenitalium produced lesions in the seminal vesicles, prostate, epididymis, testes and the pelvic urethra when inoculated into the seminal vesicles experimentally (Al-Aubaidi et al 1972; Parsonson et al 1974a). Lein & Nielsen (1975) found that both Mycoplasma bovigenitalium and Mycoplasma agalactiae var. bovis produced ascending and descending infections of the urinogenital tract when inoculated into the seminal vesicles of experimental animals. These animals shed mycoplasmas continuously in the semen and seminal fluids. In chronic infections sperm abnormalities and azoospermia also occurred. A mycoplasma, tentatively identified as M. agalactiae var bovis, when added to bovine semen prior to semen processing resulted in heifers requiring multiple inseminations before conceiving, and four failing to conceive after 5 inseminations. These animals showed chronic suppurative salpingitis, chronic endometritis and ovarian adhesions which were the cause of their sterility (Hirth et al 1966).

A chlamydial agent, Miyalawanella bovis, associated with bovine enzootic abortion, has been isolated from bovine semen (Yilmaz 1971), and an unidentified chyamydial infection produced granulous orchitis and associated abnormal spermatogenesis in a herd of bulls (Boryczko et al 1973).

Viruses (entero-like, rheo-like and papova-like) have been isolated from 17% of semen samples, with 70% isolation occurring in semen from bulls with clinical orchitis, epididymitis, balanitis or seminal vesiculitis (Branny & Zembala 1971). Thus a definite relationship between virus isolation, and clinical changes in the sex organs of bulls and low semen quality has been established. It has been postulated that such cytopathogenic agents may be responsible for non-bacterial inflammatory changes in the reproductive organs of bulls. A cytopathogenic enterovirus, which was isolated from a bull with seminal vesiculitis, produced orchitis and a temporary (3 month) arrest in spermatogenesis (Bouters 1964). Bovine ephemeral fever virus also produces temporary sterility, with head, tail and

midpiece abnormalities occurring in the sperm produced during the infection. Virus was excreted in the semen but no infection of serviced heifers was detected (Parsonson & Snowden 1974; Burgess & Chenoweth 1975). Foot-and-mouth virus can be present in semen after experimental infection and before the appearance of clinical symptoms. This virus can persist for 1 year in semen stored at -196'C, and can be transmitted by artificial insemination to serviced cows (Cottral et al 1968; Sellers et al 1968). A papova virus is associated with genital warts, producing lesions on the mucosa of the penis and/or prepuce of infected bulls (Afshar 1965). Infection with paravaccinia virus produces infertility in bulls associated with a high percentage of dead sperm and low motility among the remaining viable sperm (Johnston & Deas 1971). Parainfluenza type III virus also produces infertility as a result of degeneration of the testes following bovine infection (Deas et al 1966). Infectious bovine rhinotracheitis/ infectious pustular vulvovaginitis (IBR/IPV) virus is perhaps the most important viral agent transmitted by artificial breeding techniques. It has been isolated from the semen of a clinically normal bull in an artificial breeding centre (Spradbrow 1968); such an animal may serve as a source of infection for other breeding bulls (Bottcher & Mahler 1970). IBR virus has been isolated from the prepuce of 79% of bulls with serum neutralising antibodies to this virus (Dennett et al 1973); it has also been isolated from semen and preputial washings of bulls with epididymitis, and from nodules and discharges from cows with vaginitis (Kaminjolo et al 1975). It is released in the semen for long periods after the onset of infection, being secreted intermittently (Von Ulbrich & Haase 1974), and may be associated with low fertility in infected animals (Bwangamoi & Kaminjolo 1971). Such semen was infective after 1 year storage at -196'C (Spradbrow 1968) and heifers serviced with the contaminated semen developed histopathological evidence of endometritis, had abnormally short estrus cycles and did not conceive or had very low (13% against a normal 61%) conception rates (Kendrick & McEntee 1967; White & Snowdon 1973). Although these low conception rates are common when contaminated semen is used in artificial breeding, in natural breeding the IBR/IPV virus infective dose does not appear to affect fertility (Parsonson & Snowdon 1975).

From the above discussion it is apparent that although no correlation could be established between the number of microorganisms present in semen and either (i) the length of time that semen could be stored (Edmondson et al 1948) or (ii) the fertility level of bulls (Almquist et al 1949b), the addition of antibiotics to bovine semen prior to use in artificial breeding improved conception rates and decreased embryonic death

rates. These effects were most marked in semen from low fertility bulls (Almquist 1948; Salisbury & Bratton 1948; Almquist 1949a and b; Almquist & Prince 1950; Easterbrooks et al 1950; Foote & Bratton 1950a; Almquist 1951; Olds et al 1951; Foote & Bratton 1952; Erb & Flerchinger 1954; Willett & Ohms 1955). It has been concluded that although high microbial numbers in semen ejaculates may indicate impairment of fertility in that sample, the types of microorganisms present in semen may be of greater importance than the total number of microorganisms which can be detected.

1.5 The Value of Antibiotic Additions to Bovine Semen for use in Artificial Breeding

Artificial breeding practices basically involve two types of semen preparation. In most overseas centres, the semen is diluted, cooled and then stored at -196'C soon after collection. This method prevents bacterial growth in the extended semen although bacteria may persist for long periods of time. The addition of antibiotics to semen used in this manner helps to prevent the transmission of potential disease organisms to serviced cows.

Among the specific bovine reproductive diseases, Campylobacter (Vibrio) fetus can be eliminated from frozen semen by polymyxin B sulphate, dihydrostreptomycin, streptomycin and neomycin (antibiotics routinely added to bovine semen extenders). Exposure for several hours at room temperature prior to freezing is needed for complete effectiveness of these antibiotics. Chloramphenicol and oxytetracycline have no effect on C. fetus survival (Norman et al 1962; Adler 1965; Adler 1966; Sullivan et al 1966). In the absence of antibiotics Leptospira are known to persist in semen for three years at -196'C (Amatredjo & Campbell 1975), but penicillin and streptomycin prevent the transmission of Leptospira pomona by extended fresh semen, extended frozen semen from known shedder animals, and semen to which Leptospira organisms have been added (Sleight 1965). Mycobacterium paratuberculosis, however, can survive polymyxin B sulphate, penicillin and dihydrostreptomycin treatment (Larsen & Kopecky 1970), and Trichomonas fetus is resistant to penicillin, streptomycin and sulfanilamide (Fitzgerald et al 1954); elimination of these microorganisms from semen requires other antibiotics e.g. furazolidone or nitrofurazole (Sunaikin 1971).

Mycoplasmas in semen are also able to withstand freezing (Hirth & Nielsen 1966; Lein & Nielsen 1975), and are resistant to the usual penicillin, polymyxin and dihydrostreptomycin antibiotic additions (Hirth et al 1967). Clindamycin, lincomycin, spectinomycin and linco-spectin

inhibited mycoplasmal growth in semen (Hamdy & Miller 1971), but are not routinely used in semen extenders.

Penicillin and tetracycline are effective against Chlamydial strains isolated from cattle (Abramova et al 1974).

Several viruses with cytopathogenic effects have been isolated from bovine semen and are able to persist in semen for long periods of time, including foot-and-mouth virus (Cottral et al 1968) and IBR/IPV virus (Spradbrow 1968). Semen for use in artificial breeding should perhaps be treated to inactivate viruses especially if no serological monitoring of the animals health is maintained, and in countries, such as New Zealand, careful treatment of imported semen is essential to maintain a foot-and-mouth disease free state. Treatment with 0.2% NaOH for 10 minutes does not affect conception rates but will inactivate viral contaminants (Adler & Andersen 1970).

The second method of semen preparation is used in New Zealand during the peak period of the artificial breeding season; by this method semen is diluted and held at ambient temperatures for up to four days before being used. In this case antibiotics are particularly important in controlling the growth of microorganisms normally present in the extended semen. As discussed in CHAPTER 1.2, the addition of antibiotics to semen extenders controls bacterial growth while preserving sperm liveability (Foote & Salisbury 1948; Almquist et al 1949a; Foote & Bratton 1950b) and may improve conception rates as well as lowering embryonic mortality (Salisbury & Knodt 1947; Almquist 1948; Salisbury & Bratton 1948; Almquist 1949a; Almquist 1949b; Almquist & Prince 1950; Easterbrooks et al 1950; Foote & Bratton 1950a; Almquist 1951; Olds et al 1951; Foote & Bratton 1952; Erb & Flerchinger 1954; Willett & Ohms 1955). It has also been reported that the fertility of semen is higher, and the foetal mortality lower, after 24 hours storage than for freshly extended semen (Salisbury et al 1952; Willett et al 1955; Salisbury 1968). The enhanced fertility seen on the second and third days after collection when antibiotics have been added to the extended semen may be a function of the need for several hours of contact before the antibiotics are effective against the microorganisms present in bovine semen. In fact, microbial populations are at their lowest level at this time (Salisbury & Flerchinger 1961; Maule 1962; Brown et al 1974). The subsequent increase in total viable counts in extended semen is associated with the growth of organisms resistant to the antibiotics present in the semen extender. Brown et al (1974) found that this increase was due to yeasts which entered the extended semen in small

numbers as aerial contaminants and multiplied unchecked by the antibiotics routinely used in the semen extender. Under these conditions yeast growth seemed to determine the useful life of the semen. In other situations other organisms may be responsible for the endpoint of semen usefulness.

CHAPTER 2: THE NATURAL ANTIMICROBIAL ACTIVITY OF BOVINE SEMEN

Male reproductive tract secretions have often been reported to exhibit bacteriostatic or bacteriocidal activity. The descriptions of factors responsible for this activity suggest that several different compounds may be concerned in the various species which have been examined. Workers studying human semen have described a variety of compounds, the best known of which are lysozyme and the polyamines. Other compounds to which antimicrobial activity has been attributed are less well characterised (Rozansky et al 1949; Gurevitch et al 1951; Taylor & Morgan 1952; Razin & Rozansky 1957; Stamey et al 1968; Tallgren et al 1968; Hankiewicz & Swierczek 1974; Mardh & Colleen 1974). A similar variety of compounds with antimicrobial properties has also been reported in canine prostatic fluid (Youmans et al 1938; Stamey et al 1968; Fair & Wehner 1971b).

Gunsalus et al (1941) noted that during semen storage experiments the number of bacteria decreased in some samples. On further investigation, however, they could find no evidence of any germicidal action against Escherichia coli in bovine semen. Similarly, no activity against Staphylococcus aureus was detected in bovine semen by Gurevitch et al (1951). However, Vibrio fetus infectivity was found to decrease in bovine semen which was held above freezing point for 24 hours even in the absence of antibiotics (Willett et al 1955), and Tallgren et al (1968) showed that both human and bovine semen inhibited bacteria, although not always the same species. This has been confirmed by Brown et al (1974) and Shannon et al (1974, 1975) who noted that bovine semen had considerable antimicrobial activity against several bacterial species.

Taylor-Robinson et al (1969) showed that most bovine seminal fluids and bovine serum contain a heat stable, non-dialysable factor which inhibits the growth of Ureaplasmas. It was postulated that this factor may prevent deep-seated mycoplasmal infections of the reproductive tract but no further characterisation was attempted.

Bovine semen also has an inhibitory effect on viruses. Le Q Darcel & Coulter (1976) have confirmed an earlier report by Bitsch (1964) that seminal fluid from the raw semen of many bulls neutralises the growth of Infectious Bovine Rhinotracheitis (IBR) virus in tissue culture.

There has been no indication in the literature whether the various activities of bovine semen are associated with individual compounds or whether a single factor is responsible for its activity against bacteria, mycoplasmas and viruses. In addition there have been few attempts to identify the compound or compounds responsible for these inhibitory effects,

although recently, Shannon et al (1974, 1975) partially purified a small cationic peptide with antibacterial activity from bovine semen, which seemed to occur as polymers of varying molecular weight.

CHAPTER 3: THE AIM OF THIS INVESTIGATION

The aim of this thesis was to examine the microbial content of semen used for artificial breeding in New Zealand, and to evaluate its relationship to the natural antimicrobial activity present in bovine seminal plasma, with special reference to the possibility of disease transmission via this medium.

This study developed into an investigation of the nature and identity of the factors responsible for the antimicrobial activity in bovine semen, its mode of action and its possible role as a primary defence mechanism of the male reproductive tract.

The thesis work is divided into four parts:

In PART ONE, the microbiology of bovine semen used for artificial breeding in New Zealand is described, and its relationship with the natural antibacterial activity in bovine semen is investigated.

Once it was established that bovine semen contained considerable activity against a variety of bacteria, it was decided to investigate the properties of this activity further.

PART TWO records the chemical and physical characterisation of the inhibitory factors in bovine seminal plasma. Such characterisation proved helpful inthe comparison of bovine seminal plasma activity with the antimicrobial activity of known antimicrobial agents, and of semen from other species. It also provided information useful in the attempts to isolate the factors responsible for bovine seminal plasma antimicrobial activity.

PART THREE extends the initial observations of bovine seminal plasma's antimicrobial activity and reports on studies into the response of bacteria, mycoplasmas and ureaplasmas to bovine seminal plasma, compared with their response to known antimicrobial agents and the mode of action of bovine seminal plasma against these microorganisms.

PART FOUR describes the attempts to purify a factor responsible for at least some of the antimicrobial activity of bovine seminal plasma, the successful development of a unique isolation technique, and the preliminary characterisation of the compound isolated in this manner.

MATERIALS AND METHODS

CHAPTER 4: GENERAL EXPERIMENTAL TECHNIQUES

4.1 Semen and Seminal Plasma Samples

Bovine semen samples for (i) microbiological investigation and (ii) the preliminary survey for antimicrobial activity, were collected from bulls kept at the New Zealand Dairy Board Artificial Breeding Centre at Awahuri near Palmerston North, during the normal peak of artificial breeding use. Routine artificial breeding techniques were followed for the collection of semen using an artificial vagina fitted with a sterile test-tube (For a more detailed account see CHAPTER 1.1). Samples were transported to the laboratory within two hours of collection; the test-tubes were closed with waxed corks and held in a wallet wrapped around a bottle of ice in an insulated box; they were immediately assayed for total microbial counts (see CHAPTER 5.2) and for natural antibacterial activity against Micrococcus lysodeikticus[1] (see CHAPTER 4.3).

For further investigations into the nature of the antimicrobial activity present in bovine genital secretions, pooled seminal plasma samples from vasectomised bulls were used, to eliminate the possibility of inhibitory substances arising from the sperm cells themselves. Seminal fluid collections from these animals followed the procedure described above, and were made at the New Zealand Dairy Board Artificial Breeding Centre at Newstead, R.D. 4., Hamilton. Pooled samples were filtered through fibre-glass wool and a millipore filter to remove as many of the contaminating bacteria as possible. These samples were then frozen, airfreighted to Palmerston North, and stored at -20'C in the laboratory. Whole seminal plasma and fractions can be stored at -20'C for considerable periods of time (six months or more) without significant loss of antimicrobial activity.

4.2 Development of an Assay System to detect and measure the Antibacterial Activity in Bovine Genital Secretions

A number of biological assays were examined to select a method which could be used as a routine measure of the antibacterial activity of bovine seminal plasma. These included serial dilution in liquid or solid media as well as linear and radial diffusion assays.

FOOTNOTE: [1] Currently classified as Micrococcus luteus, Bergey (1974)

SERIAL DILUTION METHODS: Serial dilutions of membrane filter sterilised bovine seminal plasma with Ringers solution or Nutrient broth (see CHAPTER 6.1 for specifications) were inoculated with the test organism (Escherichia coli or a Bacillus sp[1]). In some cases growth of resistant organisms from the seminal plasma itself masked the inhibitory effect on the test bacterium, and although some growth inhibition of the test organism could be seen at 12% content of seminal plasma, this method was not as sensitive as other techniques described below.

For serial dilution in solidified media, membrane filter sterilised bovine seminal plasma was diluted into molten nutrient agar (for specifications see CHAPTER 6.1) poured into Petri plates and allowed to set. The test bacterium was then placed on the surface of the agar by swabbing, spreading $0.1cm^3$ cell suspension with a sterile glass spreader, or pouring 1 cm^3 cell suspension onto the agar and then draining off the excess. Spreading a known volume of bacterial suspension onto agar gives a more accurate cell population than the other less precise methods, and is desirable if close study of the effect of bovine seminal plasma on bacterial populations is required. At seminal plasma levels of 50% and 25% there was no growth of E. coli or Bacillus sp on the test plates. At 12% seminal plasma some isolated colonies appeared, and at the 3% level growth was confluent. Although less sensitive than the diffusion assay described below, this method could be of some importance in the isolation of bacteria resistant to bovine seminal plasma.

LINEAR DIFFUSION METHOD: Linear diffusion using a seminal plasma overlay on nutrient agar seeded with the test organism was not satisfactory. Heavy growth occurred at the plasma/agar interface even when diffusion was allowed for 3 hours before incubation at 37'C.

RADIAL DIFFUSION METHODS: Radial diffusion assays gave more sensitive measurements of antibacterial activity:

(a) Filter paper discs soaked in seminal plasma and then placed on the surface of nutrient agar plates streaked with a sensitive organism, gave zones of inhibition of bacterial growth to about 1mm from the disc itself.

(b) Wells cut into the agar were able to contain greater volumes of the sample to be tested and therefore gave more sensitive

FOOTNOTE: [1] Isolated by Shannon et al (1974)

responses to seminal plasma antibacterial activity. Initially two drops of seminal plasma were placed in each well using a sterile pasteur pipette, but when this method was selected as the standard assay technique a more accurate measure of 0.1cm^3 was obtained using an auto-pipette which delivers 50 microlitres at a time (see below - CHAPTER 4.3).

(c) In a more refined radial diffusion method, metal cylinders were tested as reservoirs for the test solution. In this method 3% agar was used as an underlay to prevent the cylinders from sinking into the agar. The sensitive organism was added as a second layer in 5cm^3 of molten nutrient agar (1% agar). Each cylinder held 0.3cm^3 test solution. Once these solutions were added to the reservoirs the assay was allowed to pre-diffuse 6 hours before 24 hours incubation at 37'C. This method was considerably more laborious than the agar well technique although the sensitivity was similar. It was therefore decided that the simpler method would be used for routine testing of the antibacterial activity in bovine seminal plasma (see below - CHAPTER 4.3).

(d) A "lysoplate" technique of radial diffusion using heat-killed Micrococcus lysodeikticus cells incorporated into non-nutrient agar has been described as a suitable method for the measurement of lysozyme in human secretions (Osserman & Lawlor, 1966). In this technique the action of lysozyme on the cell walls of the heat-killed bacteria produces zones of clearing about the test wells in the otherwise cloudy medium. This method, however, produced variable results when bovine seminal plasma was used as the test solution, so was not investigated further.

4.3 The Standard Method for the Assay of Antibacterial Activity in Bovine Seminal Plasma

The method for the routine testing of antibacterial activity in bovine seminal plasma used a radial diffusion technique with the test solutions held in wells punched into agar seeded with the test organism, Micrococcus lysodeikticus unless otherwise stated.

Twenty-four hour cultures of M.lysodeikticus on Brain Heart Infusion agar (Difco Laboratories, Detroit, U.S.A.) were suspended in sterile nutrient broth (Difco) and adjusted to give a reading of 80 in a Klett-Summerson

colorimeter fitted with a blue filter (400 - 460nm). 16cm^3 of sterile nutrient agar at 50'C was seeded with 0.24cm^3 of this cell suspension, poured into a petri plate 8.5cm in diameter and allowed to set. To form the wells, holes were cut in the solidified agar with a No. 4 cork borer and the plugs removed by suction. Each well was filled with 0.1cm^3 of undiluted semen or other test solution. The plates were held at 4'C for three hours to allow the diffusion of the antibacterial factors into the surrounding agar; the plates were then incubated at 37'C for 24 hours to allow growth of the seeded bacteria. The diameters of the zones of inhibition were measured with calipers to the nearest 0.25mm.

To enable the assay of large numbers of samples a large plate assay based on this system was also developed. The 'plate' used was a 25 x 25cm glass dish, sterilised by swabbing with alcohol and flaming. Metal lids were autoclaved and placed over the sterilised plates. These plates held 200cm^3 of nutrient agar seeded with a constant volume (3cm^3) of cell suspension at Klett 80 from overnight cultures of the test organism. The assay followed that described above.

Care was taken to standardise the method; it is known that the concentration and depth of the agar can effect the biological assay of antibiotics (Davis & Stout 1971). The volumes of agar used were held constant and all plates were set and incubated on a platform with adjustable legs to ensure that the plates were held level at all times. The inoculum was also standardised by adding a set volume of cell suspension with a constant optical density to any given volume of agar. The sample itself was delivered by an auto-pipette at a set volume. Prediffusion was allowed at a uniform temperature and the time measured accurately. The temperature of incubation was constant, and uneven temperatures within the plates were minimised since the plates remained on their level platform and no plates were stacked for incubation. The inhibition zones were measured as close as possible to the 24 hour incubation period stipulated.

Three samples of pooled vasectomised seminal plasma were each tested eleven times by this assay. The average inhibition zone diameter and its standard error about the mean were 16.75 $\pm$ 0.07mm, 18.16 $\pm$ 0.09 mm, and 18.00 $\pm$ 0.03mm respectively. This assay is therefore highly reproducible as a method of measuring antibacterial activity in bovine seminal plasma. In addition, other antimicrobial agents can readily be assayed under the same conditions.

4.4 The Standard Reference Assay for Comparison of the Inhibitory Activity of Bovine Seminal Plasma and Selected Antimicrobial Agents

A standard reference method was developed to allow comparison of the inhibitory activity of bovine seminal plasma and commercial antimicrobial agents, during the investigations of the physico-chemical properties of bovine seminal plasma antimicrobial activity.

This utilised the agar well diffusion technique described above (CHAPTER 4.3), employing an overnight culture of M. lysodeikticus in Brain Heart Infusion broth as the indicator organism, and a nutrient agar growth medium modified to contain 0.0225M sodium citrate (CHAPTER 14.2). For ease of comparison all antimicrobial activities were expressed as the number of "egg white lysozyme units", i.e. the number of lysozyme units, which produced an inhibition zone of equivalent diameter under the same assay conditions. (FIGURE ONE). The regression equation for the lysozyme standard curve is $y = 0.76 + 0.16x$. A similar relationship exists when bovine seminal plasma is diluted and its activity measured in the same assay system, (FIGURE ONE), with a regression equation of $y = 0.93 + 0.16x$. The regression coefficient, or slope, of the standard curves for both lysozyme and bovine seminal plasma are the same (0.16), therefore the lysozyme conversion graph can be used successfully to give a quantitative measurement of the antimicrobial activity present in bovine seminal plasma. This activity is expressed in "lysozyme unit" equivalents (LzU_e).

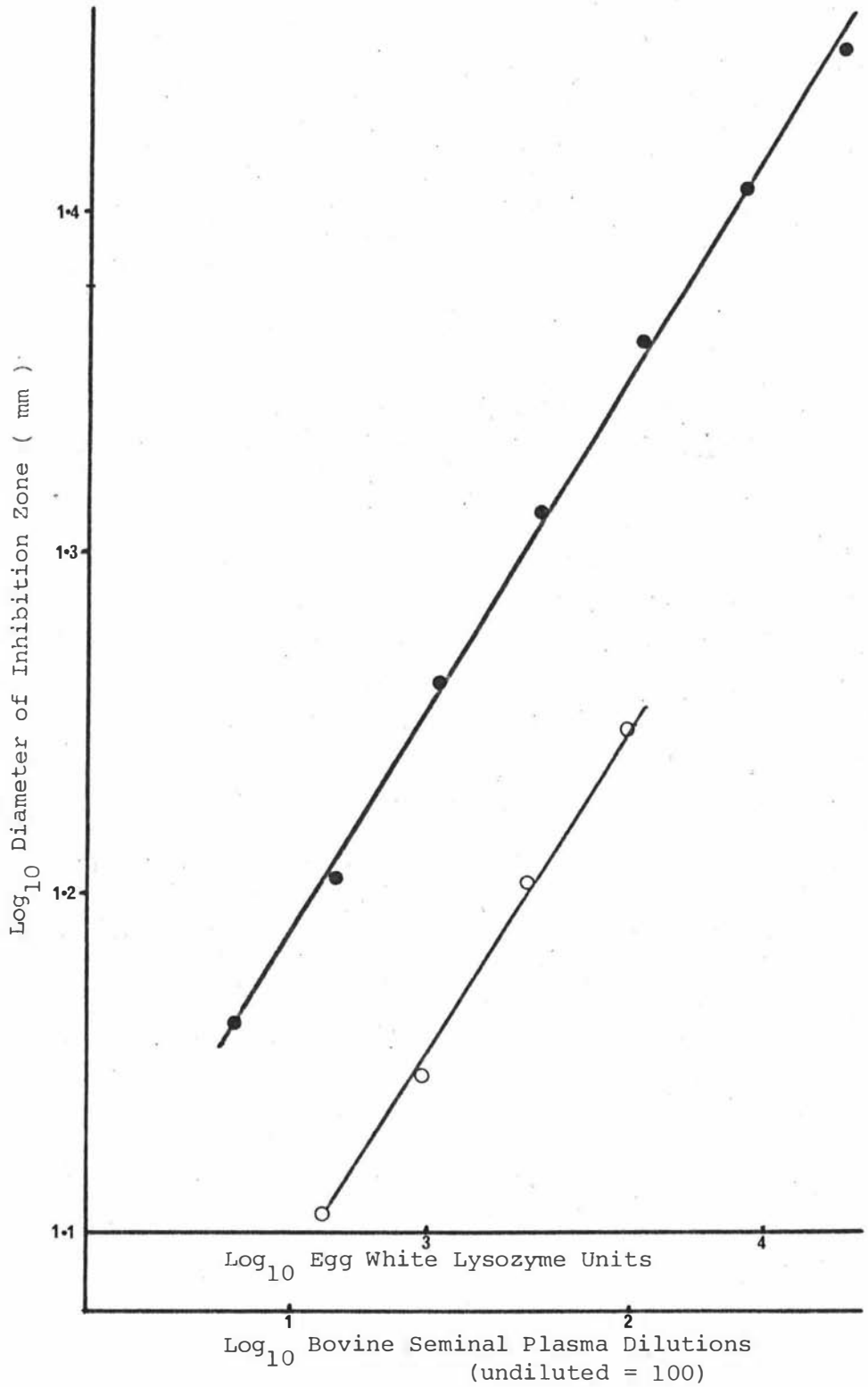


FIGURE ONE :
Standard Assay for Bovine Seminal Plasma and Egg White Lysozyme Antibacterial Activity.

● = Lysozyme (Regression Equation y = 0.76 + 0.16x)
O = Bovine Seminal Plasma (Regression Equation y = 0.93 + 0.16x)

CHAPTER 5: TECHNIQUES USED IN THE INVESTIGATION OF MICROBIAL POPULATIONS IN BOVINE SEMEN

5.1 Media used for the Determination of Total Microbial Counts in Bovine Semen

Blood-agar plates were prepared by adding, aseptically, 7% heparinised bovine blood to sterile nutrient broth (Difco Laboratories, Detroit, U.S.A.) supplemented with 0.5% sodium chloride and 1% agar (Davis Gelatine (N.Z) Ltd., Christchurch, New Zealand). The basal medium was sterilised by autoclaving for 15 minutes at 15 lb/in^2. Aliquots of approximately $16cm^3$ of this medium were then distributed into petri plates and the agar allowed to set. These prepared blood-agar plates were then incubated for 24 hours at 37'C before use, to check for bacterial contamination. Plates showing signs of bacterial growth after this period of time were discarded.

5.2 Technique for Total Viable Counts of Microorganisms in Bovine Semen

Total microbial counts on bovine semen samples were determined by spreading $0.1cm^3$ of undiluted semen, or from a 1 in 10 dilution in sterile distilled water, onto the surface of a blood-agar plate. An alcohol and flame sterilised glass spreader was used to distribute the fluid evenly over the plate. The inoculated plates were incubated at 37'C for 48 hours and then the number of colonies counted.

5.3 Materials and Method used to Test the Sensitivity of Semen Microorganisms to Commercially Available Antibiotics

The sensitivity of microorganisms isolated from bovine semen to commercially available antibiotics was tested using drug-impregnated discs containing thirteen different antibiotics. The antibiotics used, their concentration and their mode of action are recorded in TABLE I.

These discs were placed on blood-agar plates spread with the test organism and the assay was incubated for 24 hours at 37'C. The diameters of zones of complete growth inhibition about the discs were measured and each organism scored for its response to each antibiotic.

TABLE I.

ANTIBIOTICS USED TO TEST THE SENSITIVITY OF SEMEN MICROORGANISMS TO COMMERCIALLY AVAILABLE ANTIBIOTICS[1]

(a)	(b)	(c)	(d)
Antibiotics affecting Cell-Wall Synthesis	Antibiotics affecting Cell Membranes	Antibiotics Affecting Protein Synthesis	Antibiotic acting as a Metabolic Analogue
Bacitracin (10 units)	Colistin sulphate (10mcg)	Chloramphenicol (30mcg)	Sulfafurazole (250mcg)
D-cycloserine (50mcg)	Nystatin (100 units)	Erythromycin (15mcg)	
Penicillin (2 units)	Polymyxin (100 units)	Kanamycin (30mcg)	
Ristocetin (30mcg)		Streptomycin (25mcg)	
		Tetracycline (10mcg)	

FOOTNOTE: [1] Concentration per disc in parentheses.

5.4 Method for Differentiating Semen Microorganisms Sensitive to or Resistant to the Natural Antimicrobial Activity in Bovine Seminal Plasma

To differentiate between those microorganisms which were sensitive and those which were resistant to the natural antimicrobial activity present in bovine seminal fluid, colonies from the total count plates (CHAPTER 5.2) were classified into groups on the basis of morphological types. Observed differences in size, colour and morphology placed the colonies in separate categories. The number of colonies in each group was recorded and then a representative sample within each group was streaked onto fresh blood-agar plates using a sterile cottonwool swab and sterile nutrient broth as a wetting solution. One drop of filter-sterilised vasectomised seminal plasma was added to the central region of each streak. The plates were incubated at 37'C for 48 hours. Each streak and hence each microbial group was then scored as either resistant or sensitive to the antimicrobial activity of bovine seminal fluid on the basis of whether the organism grew within the region covered by the seminal plasma. Since for each total count all microbial types were counted and tested for their response to vasectomised seminal plasma, the relative numbers of resistant and sensitive micro-organisms present in semen at the time of the total count assay could be estimated.

CHAPTER 6: MEDIA AND METHODS USED IN THE IDENTIFICATION OF SEMEN MICROORGANISMS

Forty-five colonies characteristic of those commonly occurring in semen cultures were selected as representative of those microorganisms usually isolated from bovine semen. Their identification was based on the eighth edition of Bergey's Manual of Determinative Bacteriology (Bergey 1974).

6.1 Media for General Microbiological Investigations

Media in dehydrated form was reconstituted according to the manufacturers specifications, and solidified with 1.0% agar (Davis Gelatine (N.Z.) Ltd., Christchurch, New Zealand), where a solid medium was required. All media was sterilised by autoclaving for 20 minutes at 120'C, unless otherwise stated.

6.1a Blood Agar plates were prepared by adding, immediately before pouring, 7% heparinised bovine blood to sterile nutrient broth supplemented with 0.5% NaCl and 1.0% agar.

6.1b Brain Heart Infusion Broth, dehydrated medium (Difco Laboratories, Detroit, U.S.A.).

6.1c Glucose - Phosphate - Peptone Broth was prepared by adding 5g peptone, 5g glucose and 5g K_2HPO_4 to 1 litre of distilled water.

6.1d Nutrient Broth, dehydrated medium (Difco Laboratories).

6.1e Peptone - Meat Extract Broth was prepared by adding 10g peptone, and 3g beef extract to 1 litre of distilled water.

6.1f Peptone Water contained 10g peptone and 5g NaCl in 1 litre distilled water.

6.1g Ringers Solution. Tablet form. (Oxoid Ltd., London, U.K.).

6.1h Tryptone - Yeast Extract Agar, contained 10g tryptone (Difco Laboratories), 1g yeast extract and 15g agar in 1 litre of distilled water, pH 7.2.

6.2 Media and Methods for Specific Biochemical Tests

The media and methods used for specific biochemical tests during the identification of semen microorganisms followed those described by Skerman (1967), Blair et al (1970) and Cruickshank (1969).

6.2a Acid - Fastness was tested by Ziehl - Neelsen technique (Skerman 1967) on cells from an 18 - 24 hour blood agar culture at 37'C.

6.2b Catalase Test, Colonies from an 18 - 24 hour nutrient agar culture at 37'C were tested for the production of oxygen and water from hydrogen peroxide to detect the presence of catalase (Skerman 1967).

6.2c Carbohydrate Utilisation Medium. The organisms to be tested grew poorly in the basal mineral salts medium of Pope & Skerman (Skerman 1967), therefore a richer peptone broth was used (Cruickshank 1969). This broth base consisted of 5g meat extract, 10g peptone, 3g NaCl, 2g Na_2HPO_4 and 12cm^3 of bromothymol blue indicator in 1 litre of distilled water. The bromothymol blue indicator contained 1g bromothymol blue and 25cm^3 0.1N NaCl in 475cm^3 distilled water.

This medium was sterilised in test-tubes containing inverted Durham tubes. The carbohydrates to be tested (fructose, galactose, glucose, lactose, maltose, salicin) were added separately from 10% sterile solutions to a final concentration of 0.5%.

The completed media were inoculated from the growth on 24 hour blood agar plates and incubated at 37'C. Each tube was scored for acid (bromothymol blue indicator turned yellow) and gas (bubbles collected in inverted Durham tube) production after 1, 2, 3 and 6 days incubation.

6.2d Cellulolytic Activity. A strip of Whatman's No. 1 filter paper was placed in a test-tube containing peptone water and autoclaved (Skerman 1967). This broth was then inoculated with the test organism and incubated at 37'C for 2 weeks. The filter paper disintegrated when the test organism was cellulolytic.

6.2e Colony Morphology. Descriptions of the colony appearance of the microorganisms isolated from bovine semen were obtained from blood agar plates at 24, 50 and 70 hour incubation times at 37'C.

6.2f Davis & Mingioli Basal Medium, was used to test the ability of semen microorganisms to grow in a glucose minimal salts medium devoid of nitrogen compounds or with ammonium sulphate as the sole nitrogen source. The basal medium contained 20cm^3 sterile glucose solution (10%) added to a separately sterilised mixture of 7g K_2HPO_4, 3g KH_2PO_4, 0.5 g sodium citrate and 0.1g $MgSO_4$ in 1 litre of distilled water. For the medium containing nitrogen compounds 1g $(NH_4)_2SO_4$ per litre was added (Cruickshank 1969).

Preliminary growth of the test organisms was undertaken in nutrient broth at 37'C for 24 hours. The cells were then spun down at 4,000 rpm in

a Sorvall bench centrifuge, washed twice in Davis & Mingioli basal medium minus N compounds and resuspended in the same medium. These suspensions were used to inoculate test-tubes of basal medium and basal medium plus ammonium sulphate, which were then incubated at 37'C. Turbidity was used as a measure of growth and the tubes were inspected at 1, 2, 4, 6 and 7 days. After the final reading each tube was checked for purity by Gram staining the culture (see 6.2j) and examining the preparation for contaminating bacteria.

6.2g Eosin - Methylene Blue Agar (Difco Laboratories). Escherichia - type colonies possessed a metallic sheen, colorless peripheries and dark purple to black centres. Klebsiella - type colonies were larger, mucoid, brownish pink centred with a pink periphery.

6.2h Gelatine Hydrolysis. 10% gelatine (BBL, Division of Becton, Dickinson & Co., Cockeysville, Md. 21030, U.S.A.) was dissolved in nutrient broth (Difco Laboratories) and sterilised in screw-capped universal bottles. These were inoculated and incubated at 37'C for 2 weeks. After incubation the bottles were cooled at 4'C. Hydrolysis of the gelatine resulted in a liquifaction of the contents and no gel formation on cooling the medium. (Cruickshank 1969).

6.2i Glucose Oxidation/Fermentation Test, followed the recommendations of the Subcommittee on Micrococcus and Staphylococcus (1965). Tryptone - Yeast Extract agar was used for the initial growth of the test organisms. The test medium consisted of 10g tryptone (Difco Laboratories), 1g yeast extract, 10g glucose, 0.04g bromocresol purple and 2g agar in 1 litre of distilled water. This medium was autoclaved in long test-tubes, and just prior to use they were steamed for 15 minutes to remove any dissolved oxygen; they were then solidified rapidly in iced water. Heavy, deep inoculation was followed by the addition of a 2.5mm layer of sterile paraffin oil to the top of the agar, and incubation for 5 days at 37'C. The production of acid throughout the tube caused the bromocresol purple to become yellow, and indicated the ability of the test organism to ferment glucose. No acid production or the production of acid limited to the top of the tube, indicated that the test organism could only utilise glucose oxidatively.

6.2j Gram Stain was performed on smears from 18 - 24 hour blood agar cultures incubated at 37'C (Skerman 1967).

6.2k Litmus Milk (BBL). Overheating and the resultant caramelisation was avoided by fractional steam sterilisation for 30 minutes on 3 consecutive days. Various results could be detected after incubation for 48 hours at 37'C,

including acid (medium pink) or alkaline (medium blue) reactions, the reduction of litmus (medium white), peptonisation (medium watery), protein coagulation, gas production and slime formation.

6.2l MacConkey Agar (BBL). On this medium lactose fermenters produced pink colonies due to the presence of the neutral red indicator, and non-lactose fermenters produced clear to translucent colonies. Most non-enteric microorganisms were inhibited by the bile salts and crystal violet incorporated into this medium.

6.2m Methyl Red Test. Production of acid from glucose was detected in 4 day old glucose - Phosphate - peptone broth cultures (see CHAPTER 6.1c) by the addition of a solution of 0.1g methyl red in 300cm^3 95% ethanol and 200cm3 distilled water (Skerman 1967). A positive reaction resulted in a red coloration of the broth culture, indicating that the test organism metabolised glucose to acid end-products.

6.2n Motility, was tested by the direct microscopic examination using the hanging drop method of 18 hour nutrient broth cultures (Skerman 1967). Results were confirmed in semisolid nutrient agar cultures containing 0.135% agar. These cultures were read after 6, 24, 48, 72 and 144 hours incubation at both 25'C and 37'C (Cruickshank 1969).

6.2o Slide Culture. A sterile cover slip was embedded into Brain Heart Infusion Agar at about a 30' angle. The microorganisms were inoculated along the point of contact between the agar and the glass, and growth allowed for 3 days at 37'C. The cover slip was then removed and the cells adhering to it stained with methylene blue and examined under the microscope for evidence of fragmentation and conidia production (Williams & Cross 1971).

6.2p Urease Test. The basal medium (pH 6.8 - 6.9) contained 1g peptone, 5g NaCl, 2g KH_2PO_4, 1g glucose, 0.012g phenol red and 1.0% agar in 1 litre of distilled water. 100cm^3 of 20% urea solution, sterilised by filtration, was added to 1 litre of autoclaved basal medium, and the agar allowed to set as slope cultures. These slopes were inoculated with the test organisms and incubated for up to 7 days at 37'C. A positive urease test was indicated by the medium turning pink, due to an increase in the alkalinity of the medium, associated with ammonium production (Skerman 1967).

6.2q Voges - Proskauer Test. A 4 day old glucose - phosphate - peptone broth culture of the test organism was made alkaline by the addition of a few drops of 10% KOH, and then approximately 0.1cm^3 of 5% alcoholic alpha-naphthol was added. A pink coloration indicated the presence of acetyl methyl carbinol or acetoin as the end product of glucose metabolism (Cruickshank 1969).

CHAPTER 7: TECHNIQUES USED IN THE CHEMICAL AND PHYSICAL CHARACTERISATION OF THE INHIBITORY FACTORS IN BOVINE SEMINAL PLASMA

7.1 Materials used to Compare the Characteristics of Bovine Seminal Plasma Antimicrobial Activity with those of Selected Antimicrobial Agents

The Bovine Seminal plasma used during these investigations is described in CHAPTER 4.1.

Known antimicrobial agents were also examined for comparison with bovine seminal plasma antimicrobial activity. These were:

egg white lysozyme (grade 1, Sigma)

polylysine (type 2, Sigma)

salmine sulphate (British Drug House Chemicals Ltd., Poole, England)

and, spermidine phosphate (Sigma).

Lysozyme, polylysine, and salmine were tested at concentrations of 5mg/cm^3 in distilled water. Spermidine was found to exhibit lower antibacterial activity against M. lysodeikticus (APPENDIX FIVE) and was therefore used at 20mg/cm^3.

7.2 Method for the Investigation of the Effect of Proteolytic Enzymes on the Antibacterial Activity of Bovine Seminal Plasma and Selected Antimicrobial Agents

The enzymes used to determine the sensitivity of bovine seminal plasma antibacterial activity to proteolysis were subtilisin (Sigma Chemical Co., P.O. Box 14508, St. Louis, Missouri 63178, U.S.A.), trypsin (Difco Laboratories), and thermolysin (Sigma). One volume of the enzyme solution (8mg/cm^3 in pH 7.5 phosphate buffer) was added to an equal volume of bovine seminal plasma or antimicrobial agent solution, and held at 40'C for 30 minutes to permit proteolysis. The added enzymes were then inactivated by maintaining the test solutions at 60'C for 30 minutes. After cooling on ice all samples were assayed (see CHAPTER 4.4) for inhibitory activity against M. lysodeikticus.

7.3 Method for the Investigation of the Effect of Periodate Treatment on Bovine Seminal Plasma Antibacterial Activity and the Activity of Selected Antimicrobial Agents

Periodate sensitivity was tested by incubating equal volumes (0.25cm^3) of bovine seminal plasma, lysozyme, polylysine, salmine and spermidine, and 0.01M potassium periodate at 4'C for 18 hours. The excess periodate was then neutralised by adding 0.2cm^3 sterile glycerol and heating at 56'C for

30 minutes. This treatment follows that described by McFerran (1962a). All samples were then cooled on ice and assayed for antibacterial activity (Method described in CHAPTER 4.4).

7.4 Ultrafiltration Technique

Ultrafiltration was carried out with an Amicon ultrafiltration unit (Amicon Corporation, 21 Hartnell Avenue, Lexington, Massachusetts 02173, U.S.A.) under 25 psi oxygen-free nitrogen at 4'C. The membrane used (XM 50) is considered to retain molecules with a molecular weight of more than 50,000. All manufacturers instructions for handling the membrane and the apparatus were followed closely (Amicon Publication 400-A, Ultrafiltration and Diaflo Membranes for laboratory and clinical uses, 1969).

7.5 Native Polyacrylamide Gel Electrophoresis

Native polyacrylamide gel electrophoresis was performed in a vertical slab apparatus containing approximately 30cm^3 of 7.5% acrylamide and 0.15% N,N methylene bis acrylamide in O.1M Tris (hydroxlmethyl) aminomethane/ Glycine buffer at pH 8.6 (Reid & Bieleski 1968). Polymerisation of the acrylamide was induced with ammonium persulphate and N,N,N',N' tetramethyl ethylene diamine. The buffer used for running the gel was O.1M Tris/Glycine buffer pH 8.6. Once the samples, which were weighted with 40% sucrose, were loaded onto the gel, a current of 10mA was applied for 10 minutes to settle the samples into the gel, after which the current was increased to 20mA for separation of the proteins present in the samples. Bromophenol blue was used to indicate the time which small molecular weight molecules required to migrate the full length of the gel (approximately 1.5 hours). Electrophoresis towards the anode or towards the cathode was possible by altering the connections of the electrodes to the power source.

Once the sample was electrophoresed, protein bands were stained with 0.025% Coomassie Blue in a water : acetic acid : methanol solution at a ratio of 68 : 7 : 25 parts each. After staining the gel was destained with, and the gel maintained,in the same water : acetic acid : methanol solution.

7.6 Location of Antibacterial Activity in Protein Bands Separated by Native Polyacrylamide Gel Electrophoresis

Electrophoresis of the test solutions was carried out as described in CHAPTER 7.5. Once this was completed the gel was placed in a large sterile petridish, and non-nutrient agar was poured around the gel to build up the surrounds to form a level surface. This enabled a uniform layer of the sensitive test organism to be placed over the entire gel. The overlay

consisted of 25cm^3 of molten nutrient agar seeded with 0.3cm^3 of a nutrient broth suspension of Micrococcus lysodeikticus giving a reading of 50 on a Klett - Summerson colorimeter fitted with a blue filter (400 - 460nm). (FIGURE TWO). The agar was allowed to set, the plate prediffused for three hours at 4'C to allow the antibacterial factors to diffuse out in the gel and into the assay agar, and then incubated for 24 hours at 37'C to enable the test organism to grow in the assay medium. The resultant assay plate exhibited zones of bacterial inhibition in those regions where the antibacterial activity had been distributed in the gel during electrophoresis (PLATE ONE).

= Polyacrylamide gel
= Non-nutrient Agar
= Protein with no Antibacterial Activity
= Antimicrobial Protein
= Bacterial Growth in Seeded Nutrient Agar
= No Growth i.e. Inhibition of Bacteria in Seeded Nutrient Agar.

FIGURE TWO : Schematic Representation of the Sideview of the Overlay Technique for the Detection of Antibacterial Activity in Material having undergone Native Polyacrylamide Gel Electrophoresis.

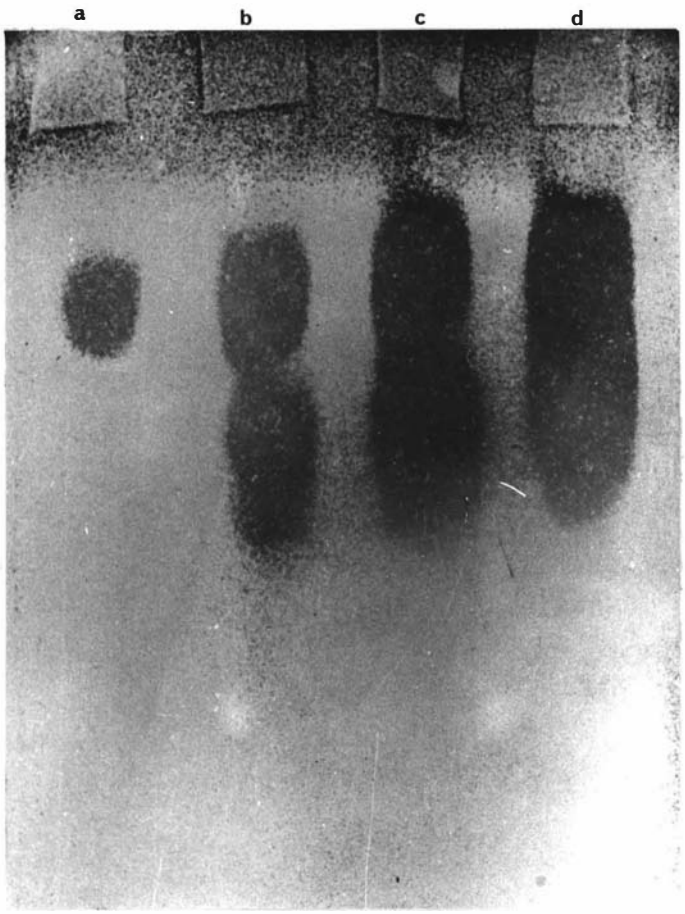


PLATE ONE : Inhibition Zones produced by Electrophoresed Cationic Proteins from Bovine Seminal Plasma (1% acetone precipitated acid dialysate, CHAPTER 18.1) after the Native Polyacrylamide Gel (pH 8.6) was overlaid with nutrient agar seeded with Micrococcus lysodeikticus.

(a) 5 microlitre sample
(b) 15 microlitre sample
(c) 30 microlitre sample
(d) 45 microlitre sample

CHAPTER 8: TECHNIQUES USED TO DETERMINE THE SPECTRUM OF ANTIMICROBIAL ACTIVITY OF BOVINE SEMINAL PLASMA

8.1 Materials used to Compare the Activity Spectrum of Bovine Seminal Plasma with those of Human Seminal Plasma and Selected Antimicrobial Agents

Bovine seminal plasma was collected from vasectomised bulls as reported in CHAPTER 4.1, and human seminal plasma was obtained from a healthy vasectomised volunteer.

The selected antimicrobial agents tested in conjunction with bovine and human seminal plasmas (lysozyme, polylysine, salmine and spermidine) are described in CHAPTER 7.1.

8.2 Techniques for Determining the Bacterial Sensitivity Spectrum of Bovine and Human Seminal Plasmas and Selected Antimicrobial Agents

The bacteria used to determine the sensitivity patterns of micro-organisms to bovine and human seminal plasmas and to selected antimicrobial agents are listed in TABLE II.

These cultures were maintained on brain heart infusion agar plates held at 4'C after 24 hours incubation at 37'C and subcultured every 14 days.

The sensitivity of each bacterium was determined by the radial diffusion technique described in CHAPTER 4.4. For those test organisms which grew poorly in citrated nutrient agar, 10% calf serum was added to the assay medium. This did not affect the sensitivity of M. lysodeikticus to bovine seminal plasma and was therefore considered a suitable addition to the growth medium for more fastidious bacteria. The Haemophilus sp. tested required supplements of 10 micrograms/cm^3 hemin and 4 micrograms/cm^3 NADH. This depressed the action of spermidine against M. lysodeikticus, but the Haemophilus sp. proved sensitive to this polyamine, so its response pattern was considered comparable with the other bacteria tested.

8.3 Techniques for Determining the Sensitivity of Mycoplasmas to Bovine and Human Seminal Plasmas and Selected Antimicrobial Agents

The mycoplasmas used to determine the inhibitory effects of seminal plasma and known antimicrobial agents are listed in TABLE II.

TABLE II.

ORGANISMS USED TO DETERMINE MICROBIAL SENSITIVITY PATTERNS TO BOVINE SEMINAL PLASMA, HUMAN SEMINAL PLASMA, LYSOZYME, POLYLYSINE, SALMINE AND SPERMIDINE

Gram positive Bacteria	Gram negative Bacteria
Arthrobacter globiformis	Acetobacter sp.
Bacillus cereus	Citrobacter freundii
Bacillus cereus v. mycoides (2)	Enterobacter cloacae
Bacillus licheniformis	Escherichia coli (8)
Bacillus megaterium (2)	Escherichia dispar
Bacillus stearothermophilus	Haemophilus influenzae
Bacillus subtilis	Klebsiella aerogenes
Corynebacterium diphtheriae gravis	Klebsiella edwardsii v. edwardsii
	Klebsiella sp.
Corynebacterium diphtheriae intermedius	Neisseria flavescens
Corynebacterium diphtheriae mitis	Neisseria gonorrhoea
Corynebacterium hofmanni	Neisseria meningitidis
Corynebacterium pyogenes	Pasteurella haemolytica
Corynebacterium xerosis	Proteus mirabilis
Lactobacillus bulgaricus (2)	Proteus morgani
Lactobacillus casei	Proteus rettgeri
Lactobacillus casei v. alactosus	Proteus vulgaris (2)
Lactobacillus casei v. rhamnosus	Providence sp.
Lactobacillus plantarum	Pseudomonas aeruginosa
Leuconostoc citrovorum	Pseudomonas flourescens
Leuconostoc mesenteroides	Salmonella arizonae
Micrococcus luteus (2)	Salmonella cholerae-suis
Micrococcus lysodeikticus	Salmonella gallinarum
Mycobacterium phlei	Salmonella newington
Nocardia asteroides	Salmonella typhimurium (2)
Staphylococcus aureus (8)	Serratia marcescens
Staphylococcus epidermidis (3)	Shigella flexneri
Streptococcus cremoris	Shigella sonnei
Streptococcus equi	Vibrio anguillarum
Streptococcus faecalis (3)	Mycoplasmas
Streptococcus lactis	Mycoplasma arginini
Streptococcus mitis (2)	Mycoplasma arthritidis
Streptococcus mutans	Mycoplasma hominis (2)

/...

TABLE II. (cont'd)

Gram Positive bacteria cont'd	Mycoplasmas cont'd
Streptococcus pneumoniae	Mycoplasma ovipneumoniae (2)
Streptococcus pyogenes	Ureaplasmas (2)
Streptococcus salivaris	
Streptomyces sp.	

FOOTNOTE: Where more than one strain of the same species of bacteria was examined, the number of strains tested is given in parentheses.

Mycoplasma species were grown initially in FM_4 broth (Frey et al 1968), modified to contain 0.2% arginine hydrochloride and using phenol red as a pH indicator. The agar well diffusion technique used to detect growth inhibition by the test solutions, was carried out on plates of FM_4 medium solidified with brain heart infusion broth containing 0.8% agar. For FM_4 agar, a more concentrated broth was used, which on addition of sterile BHI/agar solution gave a medium of the usual strength (the usual dilution was 2 FM_4 broth : 1 BHI/agar solution). An aliquot ($0.1cm^3$) of mycoplasmal broth culture was swabbed onto the surface of the test plates before the wells were cut into the agar. The procedure then followed that described in CHAPTER 4.3.

Ureaplasmas were grown and tested in U_9 broth (Shepard et al 1970). In this medium growth is detected by a colour change from yellow to pink, indicating an increase in alkalinity due to the production of ammonia from urea. Thus metabolic inhibition or lack of growth is indicated by the absence of a colour change. To investigate the effect of bovine seminal plasma on ureaplasmas the growth of these organisms in U_9 medium diluted 1 : 1 and 1 : 2 with seminal plasma was compared with controls diluted with distilled water. Subculture to fresh U_9 broth after 7 days was used to determine the presence of viable ureaplasmas. Culture onto BHI agar was used to detect the presence of any contaminating bacteria.

8.4 Techniques to Determine whether Bovine Seminal Plasma is Bacteriostatic or Bacteriocidal

Overnight cultures of bacteria in brain heart infusion (BHI) broth were adjusted with fresh sterile BHI broth to give a reading of 20 in a Klett-Summerson colorimeter fitted with a blue filter (400 -460nm). This cell suspension contained approximately $1X10^7$ colony forming units/cm^3. Antimicrobial material isolated from bovine seminal plasma by adsorption onto DNA was added to the bacterial suspension to give a final concentration of $5mg/cm^3$, and the mixture held at 25'C. Aliquots of $0.1cm^3$ were removed, diluted and plated out onto BHI agar at various time intervals to determine the number of viable colony forming units still present in the sample. All plates were incubated at 37'C for 24 hours. A parallel control containing no additions was used to record normal bacterial behaviour over the time interval studied.

This assay was later modified : the BHI media described above being replaced by nutrient broth and nutrient agar.

CHAPTER 9: TECHNIQUES USED IN THE ISOLATION OF AN ANTIMICROBIAL FACTOR FROM BOVINE SEMINAL PLASMA

9.1 The Kjeldahl - Gunning Digestion and Crismer Modified Van Slyke & Hiller Procedure for Ammonia Determination; used as a Measure of the Protein Content (Microgram N/cm^3 plasma) of Crude Preparations of an Antimicrobial Compound from Bovine Seminal Plasma.

Initially the amount of nitrogen present in seminal plasma extraction samples was determined by converting nitrogenous compounds to ammonium sulphate by Kjeldahl - Gunning H_2SO_4 digestion using copper sulphate as the catalyst. These digestion samples, however, gave a yellow/orange coloration with the Chrismer modified Van Slyke & Hiller procedure for the colorimetric quantitation of the ammonia released by the digestion. On testing the reagents used in the digestion, it was found that the copper sulphate catalyst cross-reacted with the phenate - chloramine T solution used to determine the presence of ammonium salts. A substitute catalyst was therefore required for the Kjeldahl - Gunning digestion : Selenium dioxide (0.8% in H_2SO_4) proved satisfactory (Vogel 1951). The revised procedure is described below:

Kjeldahl - Gunning Digestion : 1cm^3 sample, 1 cm^3 concentrated sulphuric acid, 0.8g K_2SO_4, 0.1 cm^3 of 0.8% selenium dioxide in concentrated H_2SO_4, and 2 - 3 glass beads were placed in Kjeldahl digestion flasks and heated over microburners until white fumes were formed. The heating was continued more strongly until 30 minutes after the fumes had disappeared and the solution had cleared. The contents of the flasks were then transferred quantitatively into 100cm^3 volumetric flasks, the pH brought to neutrality with 5M NaOH (using indicator paper) and the solution was made up to the correct volume with distilled water. The Kjeldahl flasks were rinsed twice with distilled water during the transfer to ensure all the contents of the flasks were transferred.

The Crismer modification of the Van Slyke & Hiller procedure for the determination of ammonium salts in the digested samples used chloramine T in place of the hypochlorite solution in the original method (Conway 1957). 2 cm^3 of the digested sample (in 100cm^3 distilled water at neutral pH) was added to 1 cm^3 sodium phenate solution (25g phenate in a small quantity of distilled water, added to 50 cm^3 40% NaOH and diluted to 100cm^3 with ammonia-free water), and 3 cm^3 5% Chloramine T solution. These solutions were mixed well and then heated for 10 minutes in a boiling water bath. After cooling, the absorbance of the blue colour which developed was measured on a Hitachi Spectrophotometer model 101 with a tungsten lamp and the filter lever

in position W3 (600 - 900nm). The wavelength selected was 610nm since a 10 microgram/cm^3 solution of ammonium sulphate gave a plateau over this wavelength (FIGURE THREE) in a Unicam SP800 scanning spectrophotometer.

The standard curve used to determine the amount of Nitrogen in the test samples is recorded in FIGURE FOUR, using ammonium sulphate as the ammonium source.

9.2 Techniques in Ion Exchange Chromatography of Bovine Seminal Plasma

9.2a Resins Investigated during Ion Exchange Chromatography Studies of Bovine Seminal Plasma

Several different cationic resins were studied to determine which resin preferentially adsorbed the proteins associated with the antimicrobial activity of bovine seminal plasma. The resins tested were:

(a) Amberlite IR - 120 (BDH Ltd.) a strongly acidic cation exchanger with SO_3H as its functional group. Its exchange capacity is unaffected by pH, and its exchange rate is rapid. Used for the separation of peptides and amino acids.

(b) Amberlite IRC - 50 (BDH Ltd.) a weakly acidic cation exchanger, increasing in capacity with increasing alkalinity since it has a high affinity for H ions. Having COOH as its functional group, it is capable of being buffered, and almost any ratio of salt form to free acid ion can be obtained by treating the resin to a large excess of the appropriate buffer solution. Thus exchange is possible at a controlled pH. Used in the treatment of amino acids, enzymes etc.

(c) Dowex 50 - XA (BDH Ltd.) a strongly acidic cation exchanger with SO_3H functional group. pH has no effect on its exchange capacity and exchange rate is rapid. Used for the separation of peptides and amino acids.

(d) Sephadex SP c25 (Pharmacia Fine Chemicals, Uppsala, Sweden), a strongly acidic cation exchanger with a $C_3H_6SO_3H$ functional group. This resin is in a bead form with a high charge density on the surface. Very high molecular weight polymers are unable to penetrate the bead and therefore there is little non-specific adsorption. Used for peptides, small proteins and low molecular weight solutes.

(e) Whatman CM_{32} (W. & R. Balston (Modified Cellulose) Ltd., Springfield Mill, Maidstone, Kent, England), a weakly acidic exchanger, with COOH group as the functional moiety.

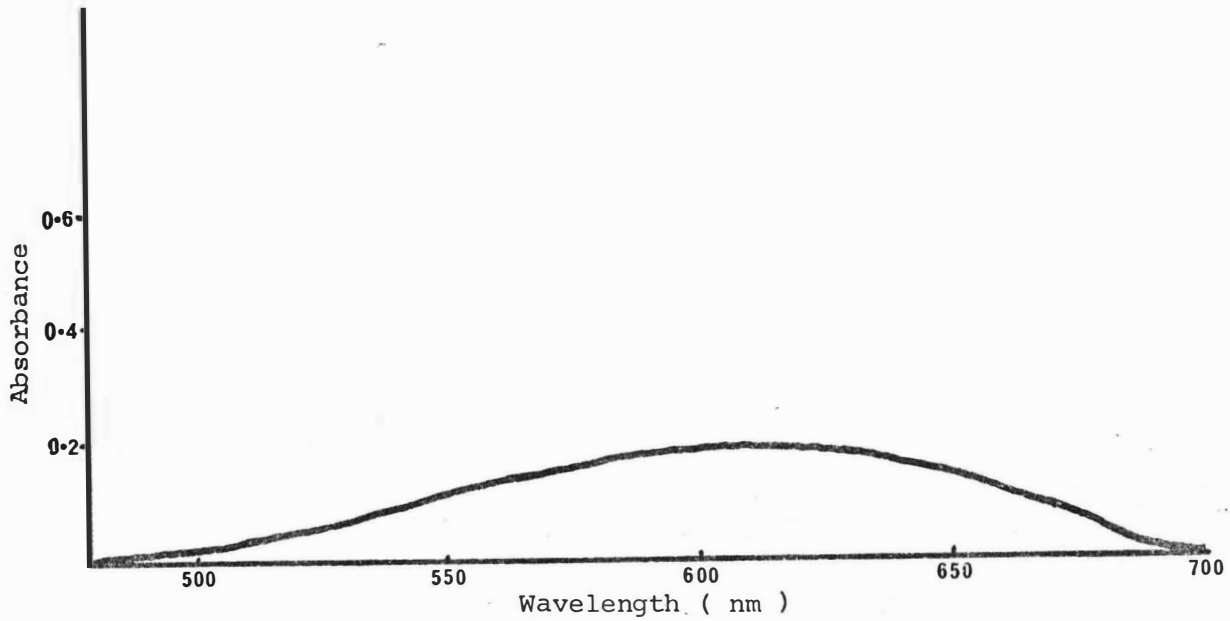


FIGURE THREE : Absorbance Spectrum (using a Unicam SP800 Spectrophotometer) of Ammonium Sulphate (10 microgram/cm^3), using the Crismer modified Van Slyke & Hiller Procedure for Ammonia Determination.

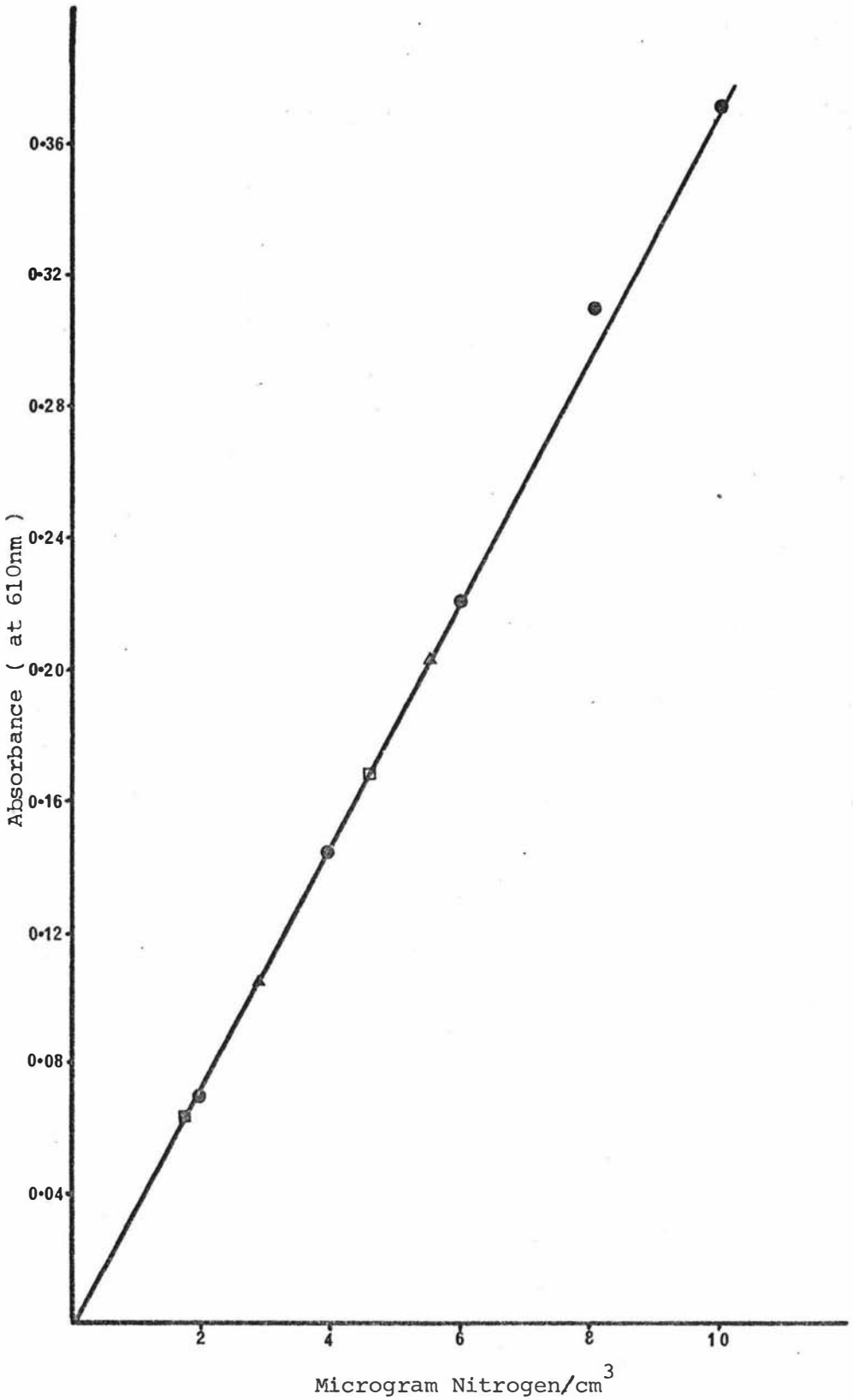


FIGURE FOUR : Standard Curve for Ammonium Sulphate in Crismer modified Van Slyke & Hiller Procedure for Ammonia Determination (used to determine the amount of ammonia present in whole, and crude extracts of, bovine seminal plasma after Kjeldahl-Gunning Digestion) .

□ = Bovine seminal plasma (1 : 20)
■ = Bovine seminal plasma (1 : 50)
▲ = Crude extract (acetone precipitated dialysate, 5mg/cm^3)
△ = Crude extract (acetone precipitated dialysate, 10mg/cm^3)

It has rapid uptake and release especially of high molecular weight biopolymers, and is kinetically compatible with large biopolymers, poly electrolytes, proteins, enzymes, etc.

9.2b Buffers used in Determining the Choice of Resin for Ion Exchange Chromatography of Bovine Seminal Plasma

The buffers tested for ion exchange chromatography were all of 0.05 molar ionic strength and within pH 0.05 of the stated pH (Dawson et al 1969). Their compositions are listed in Table III.

TABLE III.

BUFFERS USED TO DETERMINE THE OPTIMUM CONDITIONS FOR ION EXCHANGE CHROMATOGRAPHY OF BOVINE SEMINAL PLASMA

PH.	Compounds	Amounts of Constituents (All made up to 1 litre)
7.6	KH_2PO_4	42.7 cm^3 0.1M
	Na_2HPO_4	304.9 cm^3 0.05M
8.0	Tris (hydroxymethyl) aminomethane	158.4 cm^3 0.5M
	HCl	100 cm^3 0.5M
8.6	Diethylbarbituric acid	1.83g
	Sodium diethylbarbiturate	10.3g
9.0	$Na_2B_4O_7$	200cm^3 0.125M
	H_3BO_3	68.9cm^3 0.5M
9.6	$NaHCO_3$	296.4cm^3 0.1M
	Na_2CO_3	136.4cm^3 0.05M

9.2c Determination of the Most Effective Resin and the Optimum Conditions for Adsorption of the Antimicrobial Activity in Bovine Seminal Plasma

To determine the most effective resin and the optimum conditions in which the resins would adsorb the protein(s) responsible for the antimicrobial activity of bovine seminal plasma, 0.5g of each resin was pretreated according to the manufacturers specifications and then equilibrated with each of the buffers until there was no longer any change in the pH of the buffer after contact with the resin. To ensure that the Unicam Spectronic 800 absorbance scans of the treated samples after contact with the resins were not affected by the properties of the buffers themselves or any alteration occurring in the buffers from the presence of the resins, scans were made of samples of the various buffers in contact with the resins after equilibrium was reached. No significant alterations occurred at absorbances between 270 - 300nm in buffers at pH 7.6, 8.0, 9.0 and 9.6. The barbiturate buffer at pH 8.6, however, interfered with the absorbance in this range and therefore protein absorbance readings at this pH were not possible.

Once the resins were equilibrated, they were placed in centrifuge tubes and all excess moisture was removed by centrifugation at 2000 rpm followed by the discarding of the supernatants. A 6mg/cm^3 suspension of acetone precipitated material from the acid dialysis of bovine seminal plasma (see CHAPTER 18.1), in each appropriate buffer was filtered through Whatman No. 1 filter paper and then 3 cm^3 was added to each resin sample, previously equilibrated with the same buffer. These mixtures were shaken continuously for 30 minutes to allow maximum contact between resin and the antimicrobial factor(s). The supernatants were then collected by spinning down the resin at 4000 rpm for 5 minutes. The absorbances of these supernatants (diluted 1 : 3.5 in the appropriate buffer) were recorded on a Unicam SP 800 spectrophotometer, using distilled water as the reference solution and using the appropriate buffer solution to zero the instrument.

9.2d Ion Exchange Column Chromatography of a Crude Extract of the Antimicrobial Activity in Bovine Seminal Plasma

References used to gain a knowledge of Chromatographic techniques were: Sober & Peterson (1958), Morris & Morris (1964) and Heftmann (1967).

The resin used, Amberlite IRC - 50 was equilibrated with large volumes of pH 8.6 Tris/HCl buffer with ionic strength of 0.05M, until the pH of the buffer remained stable on contact with the resin. The resin/buffer mixture was then deaerated under vacuum before pouring into the column, which was

partially filled with buffer and allowed to run. This removed any air trapped in the collection well, tubing etc., and enabled the slurry to settle through a liquid when poured. A buffer reservoir was then connected to the top of the column extension and the column allowed to run for 1 hour to allow correct settling of the resin and the removal of any resin debris. When the resin was adequately settled the base runoff was closed to prevent buffer drainage and hence drying of the top of the column, and the column extension was removed. The applicator top attached to the reservoir was then placed in position on the top of the column and the column allowed to run. This ensured a final packing of the resin, and enabled a check on the equilibrium state, flow rate adjustment (approximately 16 - 20 cm^3/cm^2 column area / hour) and fraction collection programming.

The sample was acetone precipitated material (see CHAPTER 18.1) in Tris / HCl buffer, filtered through a number 1 Whatman's filter paper. The sample was added to the column with a syringe via a three way valve in the reservoir tubing leading to the top of the column. After application of the sample, the column was left to stand for 30 minutes to allow adsorption, and then the run commenced with the primary buffer, which removed nonadsorbed material from the column. The adsorbed proteins were removed from the column by stepwise elutions of increasing ionic strength (I=0.075, 0.1, 0.2, 0.5). Stepwise elution has less resolution than elution under an increasing gradient but rapid preliminary enrichment was possible. Sample volumes collected off the columns were $10cm^3$ aliquots unless otherwise stated.

The columns used were produced by Wright Scientific Ltd., England. Column I, with diameter 1.6cm and length 30cm, had a volume of $60cm^3$ and was filled with approximately 40g of resin. The sample added was $2.5cm^3$ of a $10mg/cm^3$ solution of acetone precipitated material (see CHAPTER 18.1) in Tris/HCl buffer. (N.B.: in preliminary experiments 0.5g of resin adsorbed all of the antimicrobial activity in $3cm^3$ of $6mg/cm^3$ solution i.e. 18mg precipitate. The sample added to Column I contained 25mg precipitate which could be adsorbed by 2g of resin. The optimum column functions when the top 10% of the resin is able to adsorb the applied protein. Thus this sample is well within the capabilities of this column). Column II, with diameter 2.6cm and length 20cm, had a volume of $106cm^3$ and was filled with approximately 60g of resin. The sample added was $5cm^3$ of a $6mg/cm^3$ solution of acetone precipitated material in Tris/HCl buffer. This was also well within the optimum sample size for a column of this volume. The aliquots collected during the stepwise elution from this column were $10cm^3$

samples for the first thirty tubes and then smaller (5 cm^3) aliquots were collected.

Protein absorbance of the eluates were measured at 280nm on a Unicam SP 1800 UV spectrophotometer, using a Deuterium lamp, 0.4nm slit width, 1.2nm band width, with sterile distilled water as the reference solution and the appropriate buffer to zero the instrument.

Fractions with measurable protein absorbances were assayed for antibacterial activity after concentrating to 1/10 the volume over H_2SO_4 under vacuum at 4'C. The assay procedure is described in CHAPTER 4.4.

CHAPTER 10: TECHNIQUES USED TO ISOLATE AND CHARACTERISE AN ANTIMICROBIAL FACTOR ISOLATED FROM BOVINE SEMINAL PLASMA BY ADSORPTION ONTO DEOXYRIBONUCLEIC ACID

10.1 Determination of the Optimum Conditions for Adsorption of the Antimicrobial Factor onto DNA

For these studies highly polymerised DNA (Sigma type V,Na salt from calf thymus) was used. Lower grades resulted in little recovery of DNA or active proteins.

The highly polymerised DNA was added to bovine seminal plasma samples and contact was maintained for 1 hour (unless otherwise stated) at ambient temperature with occasional shaking, to allow adsorption of the antimicrobial factor onto the DNA. Once the factor was adsorbed onto the DNA, the solubility of this complex was decreased by bringing the seminal plasma - DNA solution to 0.14M salinity. (The NaCl concentration of bovine seminal plasma is approximately 2.6mg/cm^3 (Mann 1964), a 0.14M solution contains 8.18mg/cm^3, therefore 5.58mg/cm^3 NaCl must be added to bring the seminal plasma to this salinity). The DNA - protein complex is insoluble at this concentration (Busch 1965), and can be recovered by centrifugation at 4000rpm for 20 minutes; this yields a supernatant containing the residual antimicrobial activity not adsorbed by the DNA and a pellet of the DNA - active factor complex. This pellet was washed (three times) in 0.14M NaCl to remove contaminating proteins lightly bound to the DNA. The activity of these solutions was measured by radial diffusion assay described in CHAPTER 4.4. Little antibacterial activity was present in the final wash solution.

The activity present in the supernatant and the combined washes indicated the amount of activity not strongly adsorbed onto the DNA. Thus the amount of antimicrobial activity adsorbed onto the DNA can be calculated from the total activity present in untreated seminal plasma minus the activity present in the supernatant and washes after DNA treatment, i.e. the amount was calculated by the difference.

10.2 Protein Determination by Method of Lowry et al (1951), used to measure the protein content of Material Isolated from Bovine Seminal Plasma by Adsorption onto DNA.

The final measurable color of this method results from a biuret reaction of the protein with Cu ions in alkaline conditions and the reduction of phosphomolybdic - phosphotungstic reagent by tyrosine and tryptophane in the treated protein.

The solutions needed are:

(a) 2% Na_2CO_3 in 0.1N NaOH.

(b) 0.5% $CuSO_4.5H_2O$ in 1.0% sodium or potassium tartrate.

(c) 50 cm^3 of solution(a) and 1cm^3 of solution (b). Mixed freshly each time.

(d) Folin - Ciocalteu reagent (BDH Chemicals Ltd.) diluted to 1 N acid.

The protein sample (25 - 500 microgram protein) in 1 cm^3 was mixed with 5cm^3 of solution (c) and allowed to stand 15 minutes or more. Then 0.5cm^3 of Folin - Ciocalteu reagent was added rapidly and left 30 minutes. Colorimetric readings were taken at 500nm on the Unicam SP 1800 spectrophotometer, with 0.06nm slit width and 0.2nm band width. Lysozyme was used as the standard reference protein (FIGURE FIVE).

10.3 Discontinuous Diafiltration of DNA-isolated Antimicrobial Material to Remove Citrate Ions from Solution

Discontinuous diafiltration was performed at pH 2.5, using a UM 2 ultrafilter (with neutral charge and a molecular weight retention of 1,000 daltons) in an Amicon ultrafiltration cell.

Antibacterial material can be harvested from bovine seminal plasma by adsorption onto DNA. The harvested DNA- protein complex is dissociated in the presence of citrate (2.5M), and the released DNA precipitated with 50% ethanol (CHAPTER 19). By diluting this protein - citrate - ethanol solution 1 : 10 with pH 2.5 HCl to enhance the dissociation of citrate from the antibacterial factor, and then concentrating to the original volume under 25 psi oxygen - free compressed nitrogen gas, the final concentration of the membrane permeable solutes (citrate and ethanol) was greatly reduced. This dilution and reconcentration was performed three times. The final solute concentration was

$$Sf = Si \times (d)^n$$

where Sf is final solute concentration, Si is initial solute concentration, d is the dilution factor and n is the number of times the dilution was performed i.e. $Sf = 0.25M \times (10^{-1})^3$ or 2.5×10^{-4} M.

10.4 Assay to Determine the Amount of Citrate Present in Samples of Antimicrobial Material Isolated from Bovine Seminal Plasma by Adsorption onto DNA.

The amount of citrate present in solutions of DNA-isolated material was measured by the colorimetric procedure described by McArdle (1955), using samples deproteinised by trichloroacetic acid precipitation (1 : 6.5 dilution

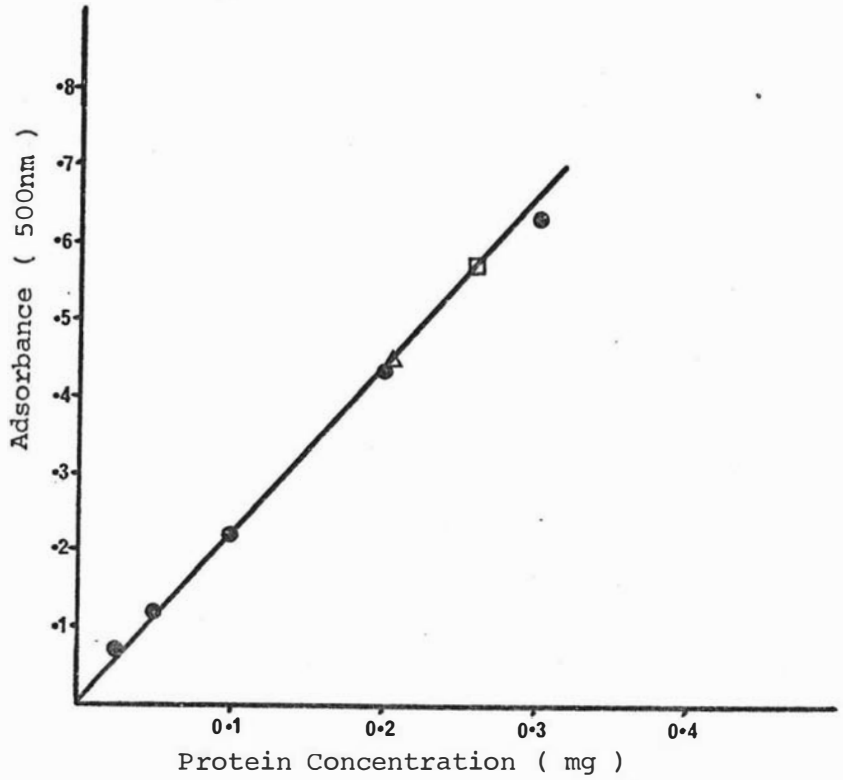


FIGURE FIVE : Standard Response Curve for the Lowry Protein Determination (Lowry et al 1951). Lysozyme used as the reference protein.

□ = Bovine seminal plasma (1 : 200)
Δ = DNA - isolated antibacterial factor (2.5mg/cm^3)

with 10% TCA solution). Deproteinised samples (5cm^3) were acidified with an equal volume of 33N H_2SO_4 and after 10 minutes, 5cm^3 of a 1.984% potassium bromide - 0.544% potassium bromate - 1.2% sodium vanadate solution was added and the mixture incubated at 30'C for 20 minutes. Traces of bromide still present were removed by the addition of 3cm^3 of 22% $FeSO_4$ in 1N H_2SO_4. The derivatives from this reaction were then absorbed into 6.5cm^3 of light petroleum, which was washed with distilled water to remove any acid traces. Any remaining water was then extracted by adding anhydrous Na_2SO_4. The color development occurred after the addition of 4cm^3 of a 4% thiourea - 2% sodium borate - 0.2% $Na_2S.\ 9\ H_2O$ solution to 5cm^3 of the petroleum extract. This combination was mixed for 1.5 minutes by bubbling with nitrogen, and then the yellow aqueous layer was pipetted into cuvettes and its absorbance at 500nm recorded. The results for the standard assays using sodium citrate are recorded in FIGURE SIX.

10.5 Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was carried out in a gel slab apparatus (Reid & Bieleski 1968) using 15% acrylamide and 0.085% N,N methylene bis acrylamide in 0.1M Tris/Glycine buffer and 0.1% sodium dodecyl sulphate. After polymerisation was complete the gel was prerun for 30 minutes at 150 volts to remove unpolymerised acrylamide and excess polymerising agents. The protein samples (10mg/cm^3) were suspended in 1.0% SDS and 1.0% beta mercapto ethanol in 0.01M Tris/glycine buffer and boiled 2 minutes before cooling and applying to the gel. Sucrose (40% w/v) was added to each protein solution to weigh down the sample, and unless otherwise stated, 30 microlitres of these solutions were added by a micropipette to each well in the polyacrylamide gel slab. Once the samples were added, the voltage was held at 40 volts for 15 minutes to settle the proteins into the gel, and then increased to 150 volts for 2 hours. After the run proteins were stained with 0.2% Coomassie blue in a solution of 5 volumes methanol: 5 volumes water: 1 volume acetic acid. Contrast was improved by destaining the surrounding gel with a solution of 4 volumes methanol: 15 volumes water: 1 volume acetic acid. Molecular weights can be calculated by the distance a protein migrates from the origin during the electrophoretic run, compared to the migration of protein standards of known molecular weights (Weber et al 1972).

The protein standards used to determine the molecular weight of the antimicrobial factor isolated from bovine seminal plasma by DNA extraction process were: Bovine serum albumin, molecular weight 136,000 & 68,000 daltons (Fluka, A.G. Chemische Fabrik. 9470 Buchs, Switzerland).

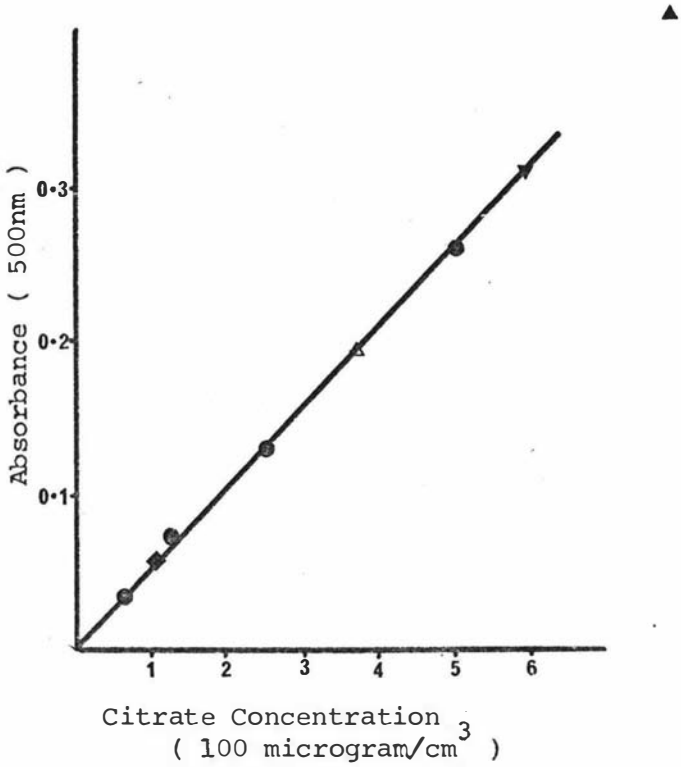


FIGURE SIX : Spectrophotometric Readings at 500nm for the Standard Citrate Assay , used to determine the amount of citrate present in antimicrobial material isolated from bovine seminal plasma by adsorption onto DNA, and to follow the removal of citrate from this material by diafiltration.

△ = DNA isolated material after diafiltration
▲ = 1st effluent after diafiltration
▼ = 2nd effluent after diafiltration
◆ = 3rd effluent after diafiltration

Lactic dehydrogenase, molecular weight 36,000 (Type V,Sigma).

Trypsin, molecular weight 23,300 (crystalised from beef pancreas, BDH).

Myoglobin, molecular weight 17,200 (Mann Research Laboratories Inc., Division of Becton, Dickinson & Co., New York, U.S.A.).

Haemoglobin, molecular weight 15,500 (Type 1, Sigma).

Lysozyme, molecular weight 14,300 (Grade 1, Sigma).

RESULTS : PART ONE

THE MICROBIOLOGY OF BOVINE SEMEN AND ITS RELATIONSHIP TO THE ANTIBACTERIAL ACTIVITY PRESENT IN THIS SECRETION

CHAPTER 11: MICROBIAL POPULATIONS IN BOVINE SEMEN

11.1 The total Microbial Populations of Bovine Semen

Semen was examined from eighteen bulls sampled during normal artificial breeding collections. Of these, four bulls (22%) were sampled only once, three (17%) were sampled 2 - 3 times and eleven (61%) were sampled more than four times. The total number of ejaculates in the survey was 111. APPENDIX ONE records the total number of viable micro-organisms present in individual semen samples collected on the days mentioned. APPENDIX TWO records similar findings for paired ejaculates collected from the same bull within a few minutes of each other.

The total number of microorganisms in semen was found to vary considerably. Individual total counts ranged from 3 x 10' to 5.6×10^5 organisms/cm^3, with a logarithmic average of 3.28×10^3 colonies/cm^3. FIGURE SEVEN records the distribution of colony counts for 111 samples of semen from 18 bulls. From this it can be seen that 52% of the semen samples contained microbial populations within the range 1.0×10^3 to 1.0×10^4 organisms/cm^3. A further 19% had populations of more than 1.0×10^4 organisms/cm^3, while 29% of the samples contained less than 1.0×10^3 microorganisms/cm^3. These variations were not due to bulls producing semen with characteristically high or low viable counts, but rather to the day to day fluctuations which occurred in the microbial numbers in semen from apparently healthy animals (FIGURE EIGHT). These day to day fluctuations in microbial populations of semen from individual bulls was reflected in both first and second ejaculate populations (FIGURE NINE).

The number of microorganisms present in semen samples from bulls of different breeds did not vary significantly. There was too great a fluctuation in the microbial populations within each breed to enable differences between breeds to be apparent. The logarithmic average of the total counts for semen from Fresian, Charolais and Hereford bulls were 3.69×10^3, 3.56×10^3, and 1.99×10^3 microorganisms/cm^3 semen respectively.

Although the wide individual fluctuations mean that the apparent difference between microbial populations in first and second ejaculates was not statistically significant, there was a consistent trend to lower

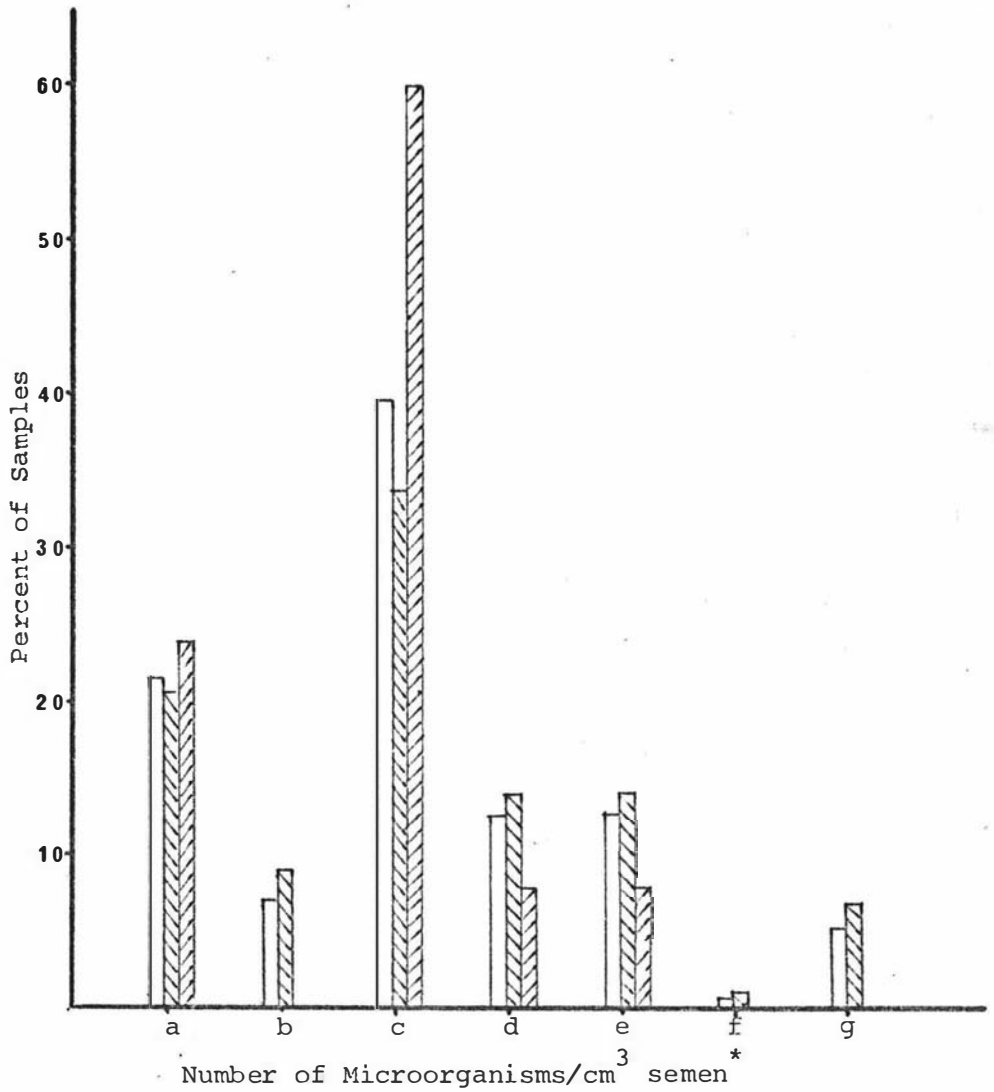


FIGURE SEVEN : The Distribution of Total Microbial Counts in Individual Bovine Semen Samples.

□ = Percent total number of semen samples
▨ = Percent first ejaculates
▨ = Percent second ejaculates

* (a), 2×10^1 to 5×10^2
(b), 5×10^2 to 1×10^3
(c), 1×10^3 to 5×10^3
(d), 5×10^3 to 1×10^4
(e), 1×10^4 to 5×10^4
(f), 5×10^4 to 1×10^5
(g), more than 1×10^5

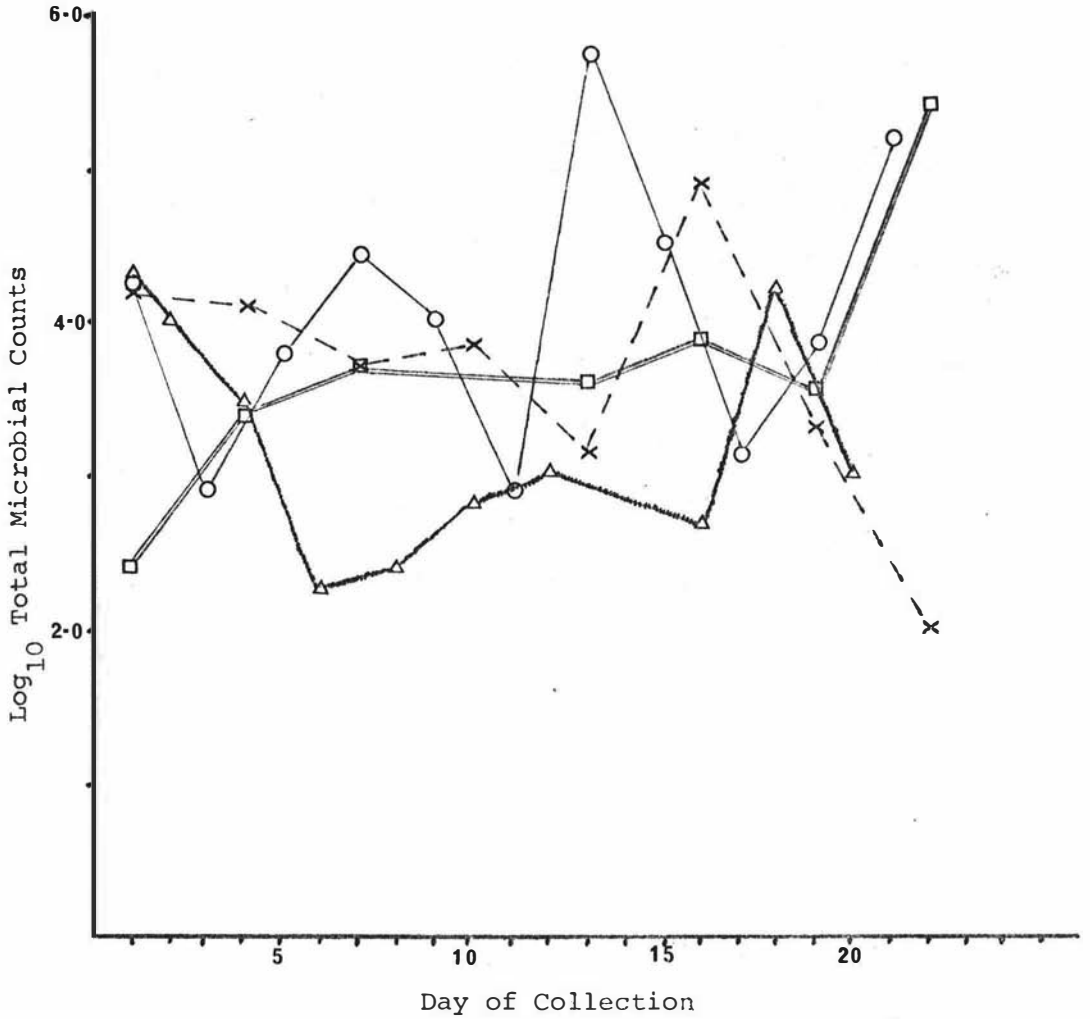
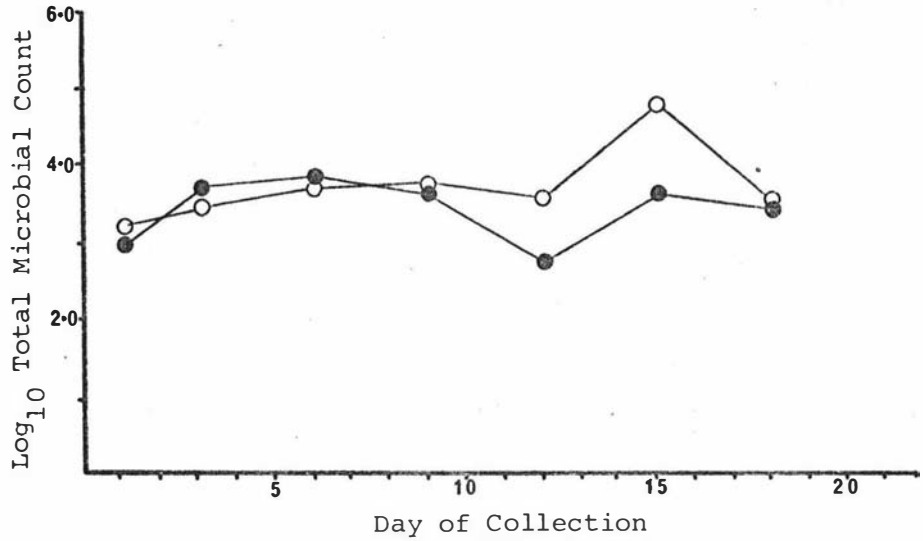


FIGURE EIGHT : Day to Day Variations in Total Microbial Counts in Semen Samples from Individual Bulls.

—O— = Charolais Bull 66076
—Δ— = Charolais Bull 66175
—×— = Fresian Bull 6200
—□— = Fresian Bull 9230

(a) Fresian, 5211



(b) Fresian, 6200

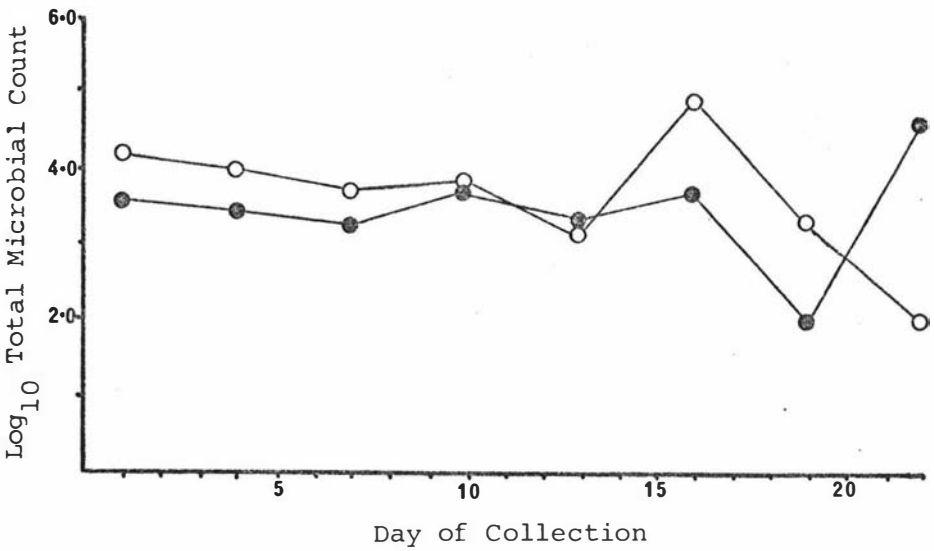


FIGURE NINE: Day to Day Fluctuations in the Microbial Populations of First and Second Ejaculates of Individual Bulls.

O= first ejaculate
●= second ejaculate

bacterial numbers in the second ejaculate when two samples were obtained within a few minutes of each other. This is similar to findings by Salisbury et al (1939), Almquist et al (1949b) and Sorel (1961). In this survey, the logarithmic average for first ejaculates in the 25 pairs examined was 3.6×10^3 microorganisms/cm^3 semen, and for the second ejaculates 1.96×10^3 microorganisms/cm^3 semen. 17 of the 25 pairs (68%) of ejaculates had higher counts in the first sample collected than in the second ejaculate. Evidence of such a lowering of the microbial population in repeated semen collections was also seen in the relative distributions of the total counts between first and second ejaculates (FIGURE SEVEN). Among first ejaculates 25% of the samples contained populations of more than 1.0×10^4 microorganisms/cm^3, compared with 8% of second ejaculate samples.

11.2 Estimation of the Variety of Microorganisms Occurring in each Semen Sample

Colonies from the total count plates were differentiated on the basis of their morphological appearance, and the number of distinguishable types recorded for each semen sample. These results are shown in column (c), APPENDIX ONE.

Cultures of bovine semen yielded from one to nine different colony types with a mean value of approximately four distinguishable colony types per semen sample (FIGURE TEN). When the microbial populations of paired ejaculates were examined the average value for the number of different microbial colony types present in the first ejaculate was 4.4, and for a second semen sample collected a few minutes after the first, the average value was 3.5. This apparent difference was statistically significant at the five percent confidence level (using the t test for the comparison of the two populations, see Bailey (1968)). A second ejaculate, therefore, contained fewer colony types than did the first sample collected on the same day.

11.3 The Types of Microorganisms Commonly Isolated from Bovine Semen

Forty-five colonies characteristic of those commonly occurring in semen cultures on blood agar plates were selected for further study. The description of each organism (APPENDIX THREE) was compiled using the techniques and media described in CHAPTER SIX. The subsequent identification of these isolates was based on the comprehensive key to the identification of genera of bacteria described in Skerman (1967). These classifications have been updated to conform with the current eighth edition of Bergey's

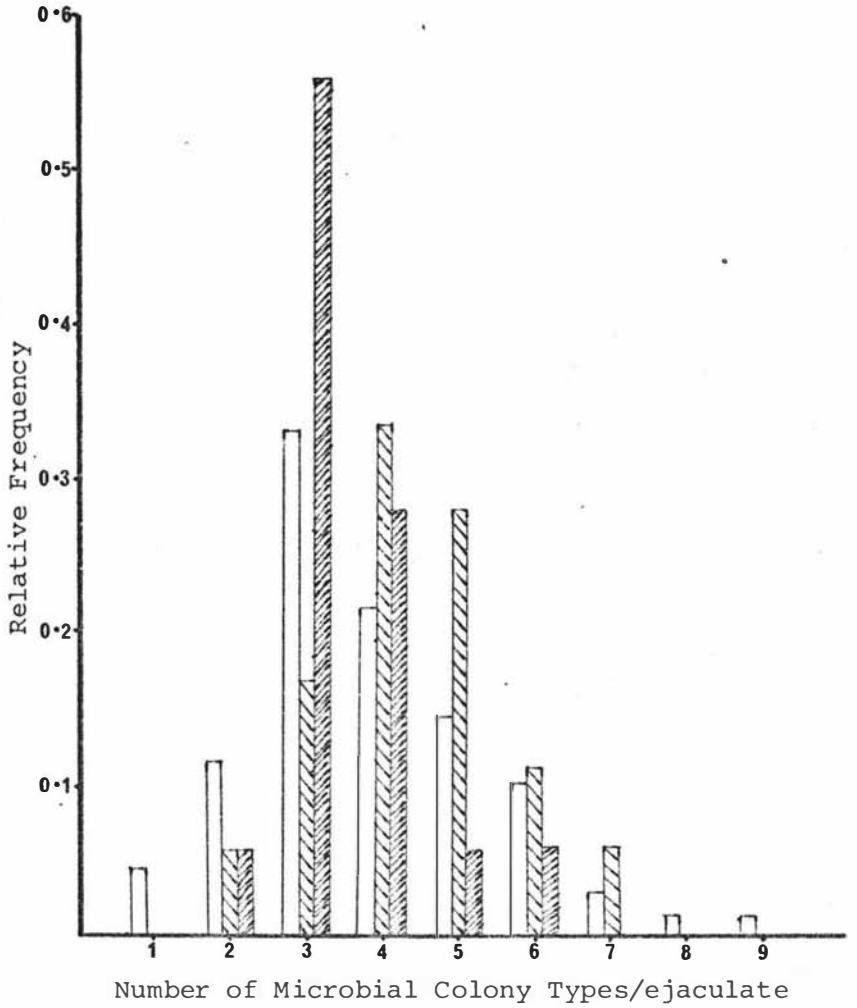


FIGURE TEN: The Relative frequency of Distribution of the Number of Different Microbial Colony Types per Ejaculate.

□ = Total Ejaculates
▨ = First Ejaculates
▨ = Second Ejaculates

Manual of Determinative Bacteriology (1974).

The most common bacterial genera represented by the selected isolates were Bacillus (9 isolates), Corynebacterium (8 isolates), Micrococcus (7 isolates), and Pseudomonas (4 isolates). Others occurring with a lower frequency were Aerobacter (2 isolates), Alcaligenes, Arthrobacter, Brevibacterium, Escherichia (2 isolates) Flavobacterium, Herellea (2 isolates)[1], Microbacterium, Nocardia, Proteus, Sarcina[2], and Staphylococcus. Two yeasts were also identified, Candida guilliermondii and Rhodotorula rubra.

Full descriptions of the morphology, colony characteristics and biochemical responses of these organisms are contained in APPENDIX THREE.

The bulls used in New Zealand artificial breeding centres are carefully monitored for infection with specific bovine venereal diseases, so it is not surprising that Campylobacter fetus, Mycobacterium paratuberculosis, Brucella abortus etc. were not isolated from the semen samples investigated.

No attempts were made to isolate viruses or mycoplasmas from bovine semen or seminal plasma.

11.4 The Antibiotic sensitivity of Microorganisms Isolated from Bovine Semen

A range of thirteen antibiotics (TABLE I) were tested against thirty-nine strains of microorganisms isolated from bovine semen. Of these, three (chloramphenicol, penicillin and streptomycin) are routinely used in semen extenders under New Zealand conditions. The response of these micro-organisms to each antibiotic were scored as being sensitive or resistant according to whether or not a zone of inhibition was produced about the antibiotic-impregnated disc in the sensitivity test (CHAPTER 5.3). The results are summarised in APPENDIX FOUR.

The proportion of strains sensitive to each antibiotic was given as an index of effectiveness, and may be used to compare the overall efficiency of different antibiotics. As can be seen from TABLE IV, the antibiotics tested varied considerably in their effectiveness. Two of the antibiotics routinely used in semen extenders, streptomycin and chloramphenicol, appeared to have satisfactory powers of antibacterial activity but penicillin was of low effectiveness.

The relative sensitivity of the microbial genera to the antibiotics is contained in TABLE V. The index of resistance represents the proportion

FOOTNOTES: 1, currently classified as Acinetobacter (Bergey 1974)
2, currently classified as Micrococcus (Bergey 1974)

TABLE IV.

THE RELATIVE EFFECTIVENESS OF SELECTED ANTIBIOTICS ON MICROORGANISMS FROM BOVINE SEMEN

Antibiotic	Number of Strains		Index of Effectiveness *
	Resistant	Sensitive	
Streptomycin	4	35	0.90
Chloramphenicol	6	33	0.85
Kanamycin	6	33	0.85
Colistin	9	30	0.77
Polymyxin	9	30	0.77
Tetracycline	9	30	0.77
Erythromycin	14	25	0.64
Ristocetin	15	24	0.62
Bacitracin	16	23	0.59
Penicillin	20	19	0.49
Cycloserine	27	12	0.31
Sulfafurazole	28	11	0.28
Nystatin	35	4	0.10

FOOTNOTE: * The proportion of bacterial strains sensitive to each antibiotic.

TABLE V.

THE RELATIVE RESISTANCE OF MICROORGANISMS ISOLATED FROM SEMEN TO A VARIETY OF ANTIBIOTICS

Genus	Number of Strains	Index of Resistance *
Candida	1	0.92
Rhodotorula	1	0.85
Pseudomonas	1	0.77
Corynebacterium	1	0.77
Pseudomonas	3	0.69
Proteus	1	0.69
Escherichia	1	0.62
Aerobacter	1	0.54
Escherichia	1	0.54
Aerobacter	1	0.46
Alcaligenes	1	0.46
Bacillus	1	0.46
Corynebacterium	2	0.38
Micrococcus	1	0.38
Arthrobacter	1	0.31
Bacillus	1	0.31
Corynebacterium	2	0.31
Microbacterium	1	0.31
Micrococcus	1	0.31
Nocardia	1	0.31
Bacillus	2	0.23
Corynebacterium	2	0.23
Micrococcus	2	0.23
Sarcina	1	0.23
Staphylococcus	1	0.23
Bacillus	3	0.15
Micrococcus	2	0.15
Bacillus	2	0.08

* The proportion of antibiotics to which each bacterial strain is completely resistant.

of antibiotics to which each strain is completely resistant (i.e. no inhibition zone produced). None of the microorganisms tested were completely resistant to all the antibiotics tested, although the yeast species were resistant to all except nystatin in both cases, and to polymyxin for Rhodotorula sp. The Gram - negative rod-shaped bacteria (Pseudomonas, Proteus, Escherichia) were considerably more resistant than the Gram - positive bacteria. Control of these bacteria is most important since they will grow at O - 25'C in extended semen (Foote & Salisbury 1948 ; Foote & Bratton 1950b). Of the antibiotics normally used in extenders streptomycin appears to be the most effective in inhibiting these bacteria. Penicillin had no effect.

Although some strains were found to be resistant to a wide variety of antibiotics, most of the organisms isolated at high frequency from semen were sensitive to the antibiotics used, so that resistant organisms are only a relatively small proportion of the population present in semen.

CHAPTER 12: THE ANTIBACTERIAL ACTIVITY OF BOVINE SEMEN

12.1 The Occurrence of Antibacterial Activity in Individual Semen Samples

When individual semen samples (CHAPTER 4.1) were tested for antibacterial activity against M. lysodeikticus by the radial diffusion agar well technique (CHAPTER 4.3), antibacterial activity was found in all ejaculates tested. The diameter of the inhibition zone produced by bovine semen ranged from 15.25 to 22.75mm (or 4,000 to 44,500 LzU_e/cm^3), with a mean of 19.39mm (or 18,083 LzU_e/cm^3) and a standard error of the mean at 0.15 mm (or 770 LzU_e/cm^3). The individual results for 111 ejaculates are recorded in APPENDIX ONE and TWO.

The activity in semen from each individual bull was relatively constant and characteristic for that animal, but there was some variation between individual bulls of the same breed (TABLE VI). The average concentration of antibacterial activity in semen from Hereford bulls, mean inhibition zone of 17.46 $\pm$ 0.22mm in diameter (or 9152 $\pm$ 622 LzU_e/cm^3), appeared to be lower than those of Fresian or Charolais bulls, which produced zones of 19.74 $\pm$ 0.16mm (or 19604 $\pm$ 918 LzU_e/cm^3) and 20.02 $\pm$ 0.28mm (or 21352 $\pm$ 499 LzU_e/cm^3) respectively. The differences in zone diameters or LzU_e values between Hereford and Fresian and between Hereford and Charolais were, statistically, highly significant (less than 1% confidence limits). The slightly higher mean activity in Charolais compared with Fresian semen was not significant at the 5% confidence limits. The relative distribution of varying levels of antibacterial activity in semen from the different breeds also showed this relationship. The modal peak for Hereford semen occurred at 5,000 to 10,000 LzU_e/cm^3 (or 18 to 19mm zone diameter), for Fresian semen at 15,000 to 20,000 LzU_e/cm^3 (or 19 to 20mm zone diameter), and for Charolais semen at 20,000 to 25,000 LzU_e/cm^3 (or 20 to 21mm zone diameter). See FIGURE ELEVEN.

There was some indication that the second ejaculate of a pair collected within a few minutes of each other from the same bull, possessed higher antibacterial activity than the first sample collected (TABLE VI AND FIGURE TWELVE). The mean value for the first ejaculate of a pair was 18046 $\pm$ 1344 LzU_e/cm^3 (or 19.49 $\pm$ 0.25mm zone diameter), whereas the mean for the second was 21094 $\pm$ 1822 LzU_e/cm^3 (or 19.92 $\pm$ 0.30mm zone diameter). This difference was not statistically significant at the 5% limit of confidence, but the modal peak of the second ejaculate, 15,000 to 20,000 LzU_e/cm^3 (or 20 to 21mm zone diameter) was greater than that of the first ejaculate, 10,000 to 15,000 LzU_e/cm^3 (or 19 to 20mm zone diameter).

TABLE VI.

THE INHIBITION OF MICROCOCCUS LYSODEIKTICUS BY SEMEN FROM SEVERAL INDIVIDUAL BULLS

Breed	Bull	Number of ejaculate	Number of Samples	Inhibition Zone Diameter(mm)	LzU_e/cm^3
Fresian	9230	1	7	18.64 ± 0.51	13,686 ± 1,682
Fresian	5211	1	7	19.50 ± 0.42	17,550 ± 1,840
		2	7	19.79 ± 0.50	19,764 ± 2,788
Fresian	6200	1	8	19.00 ± 0.35	14,838 ± 1,633
		2	8	19.60 ± 0.24	18,031 ± 1,365
Charolais	66,076	1	11	20.00 ± 0.35	20,864 ± 2,086
Charolais	66,175	1	10	19.85 ± 0.52	20,735 ± 2,740
Hereford	72,613	1	5	17.65 ± 0.40	9,630 ± 1,216
Hereford	72,624	1	5	17.30 ± 0.27	8,440 ± 731

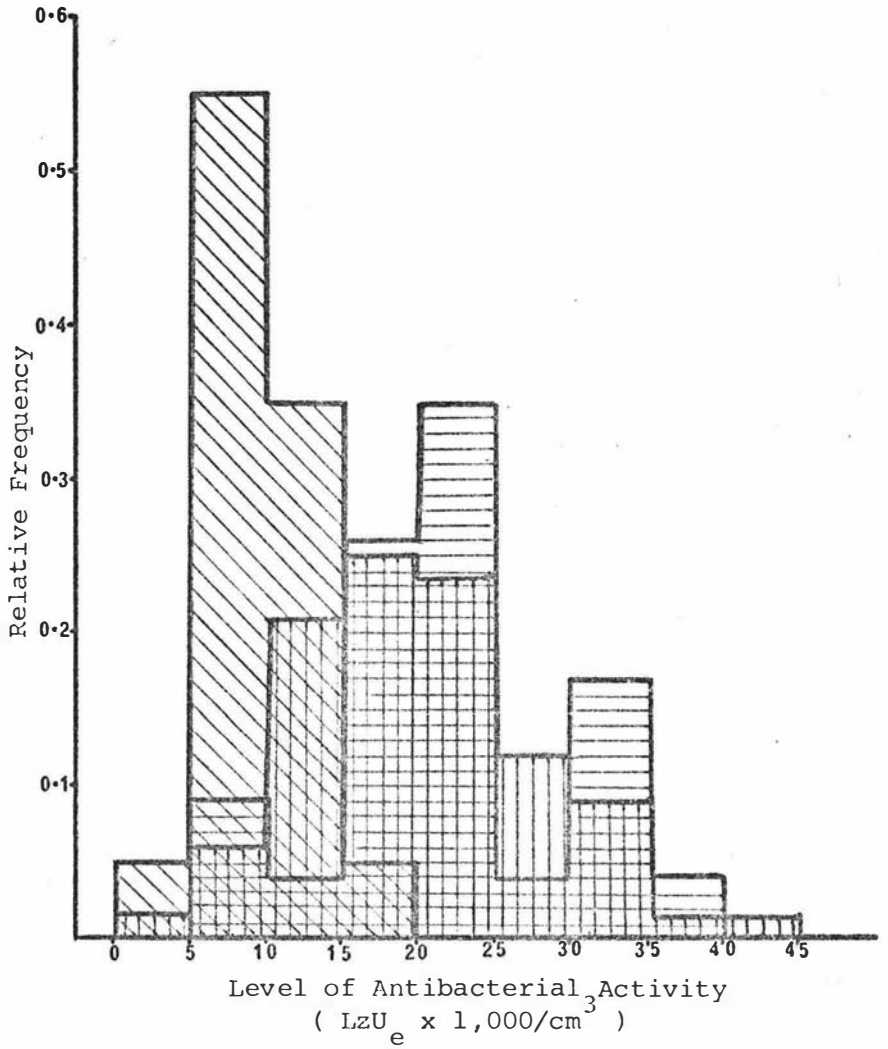


FIGURE ELEVEN: The Relative Frequency of Distribution of Different Levels of Antibacterial Activity in Semen from Bulls of Different Breeds.

= Hereford
= Fresian
= Charolais

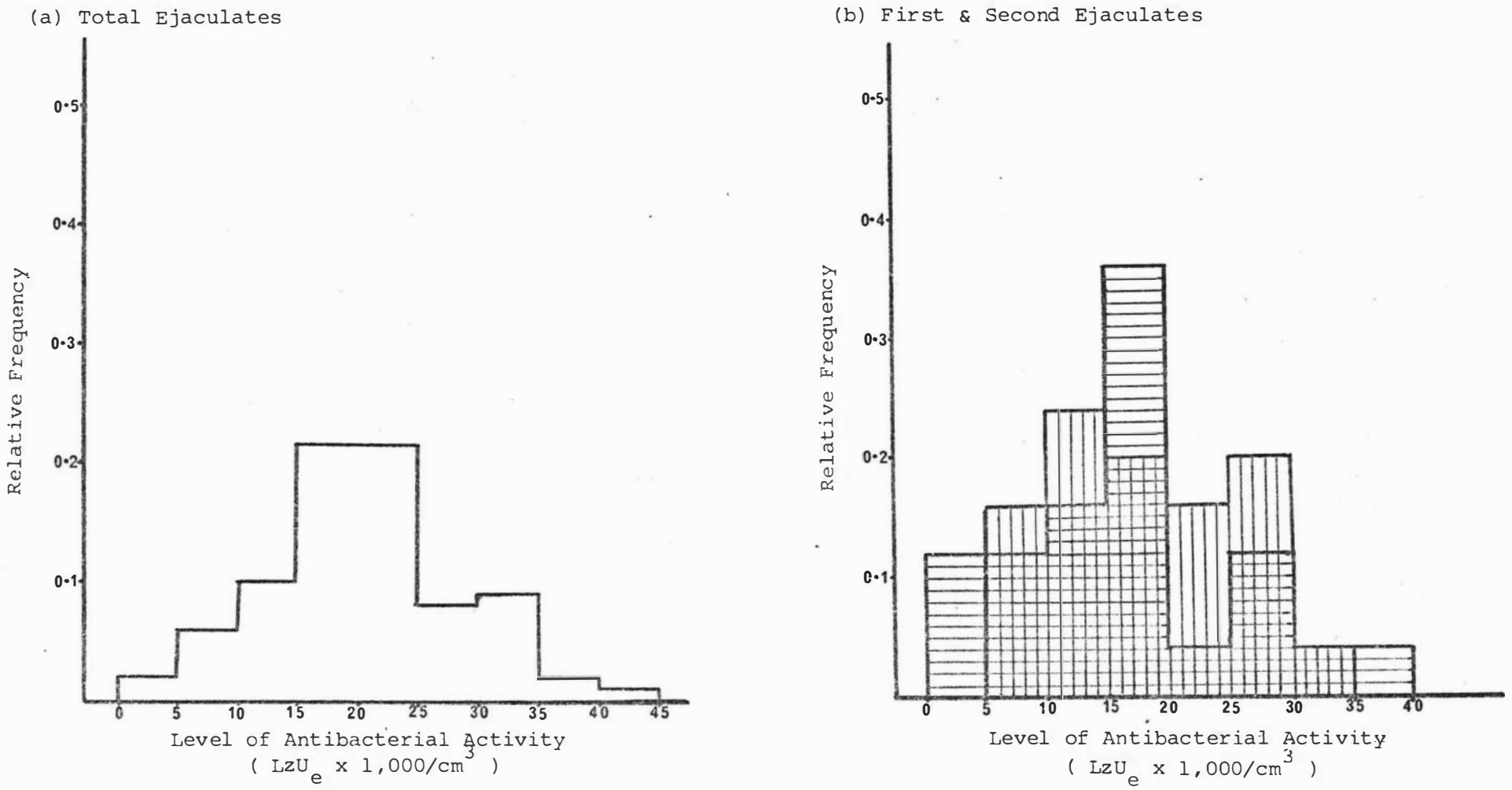


FIGURE TWELVE: The Relative Frequency of Distribution of Antibacterial Activity in (a) Total Ejaculates (b) First Ejaculates , ▥ , & Second Ejaculates, ▤ .

12.2 The Effect of the Natural Antibacterial Activity in Bovine Semen on the Total Microbial Population in Semen

The total counts of microorganisms in each semen sample and the antibacterial activity of that semen sample (recorded in APPENDIX ONE and TWO) were plotted in FIGURE THIRTEEN. The distribution of the microbial populations in relation to the amount of antibacterial activity was scattered (regression coefficient - 0.00001). There was,therefore, no correlation between the measurable concentration of the antibacterial activity in semen and the total microbial count in the same semen sample. Similarly there was little difference in the distribution of microbial populations in relation to the amounts of antibacterial activity between first and second ejaculates,with regression coefficients of - 0.00002 and - 0.00003 respectively.

12.3 The Effect of Natural Antibacterial Activity on the Number of Microorganisms Sensitive to and Resistant to this Activity in Individual Semen Samples

The microorganisms present on the total count plates from semen cultures were differentiated on the basis of their response to the antibacterial activity of bovine seminal fluid (CHAPTER 5.4) ,and the populations of microorganisms resistant to or sensitive to bovine seminal plasma were recorded for each individual semen sample (APPENDIX ONE and TWO).

There appeared to be no correlation between either the number of organisms resistant to or the number sensitive to the antibacterial activity and the concentration of measurable antibacterial activity in a given semen sample, with regression coefficients of - 0.00002 and - 0.000002 respectively (FIGURE FOURTEEN). Wide fluctuations in the populations of both sensitive and resistant organisms occurred within ejaculates collected within minutes of one another, and within samples taken from the same bull at various time intervals (FIGURE FIFTEEN and FIGURE SIXTEEN). This suggests that many of the microorganisms present in semen constitute a transient population from outside the reproductive tract.

Large (greater than 2.0×10^4 organisms/cm^3) populations of microorganisms resistant to bovine seminal plasma inhibition were found to occur in 11 individual semen samples. The population of resistant organisms in each semen sample ranged from 0 to 5.6×10^5 organisms/cm^3, with a logarithmic average of 1.4×10^3 organisms/cm^3. Although there was no

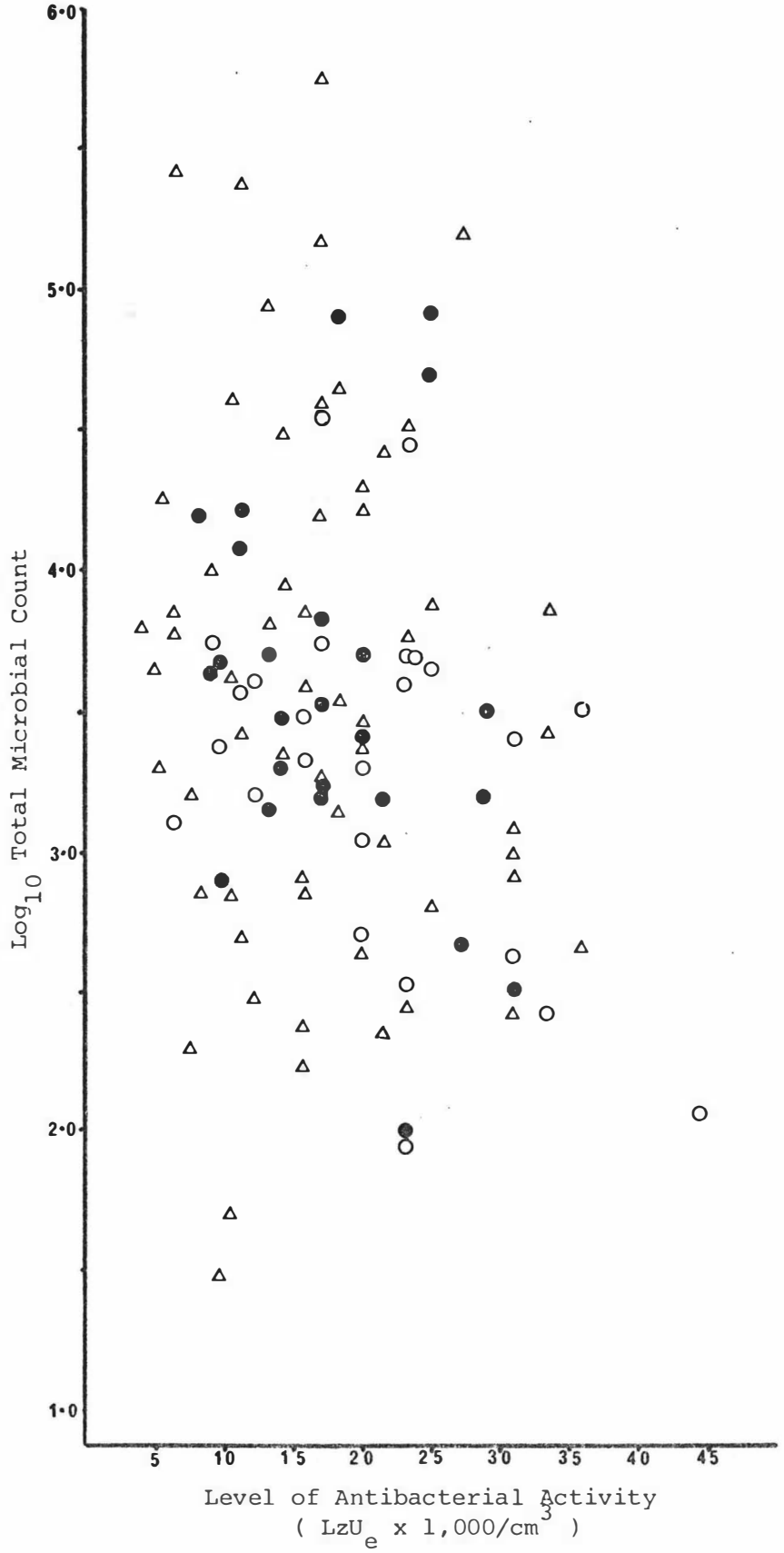


FIGURE THIRTEEN: Log_{10} Total Microbial Population Versus the Level of Antibacterial Activity in Individual Semen Samples.

● = first ejaculate of paired samples
O = second ejaculate of paired samples
△ = first ejaculate of individual bulls (non paired)

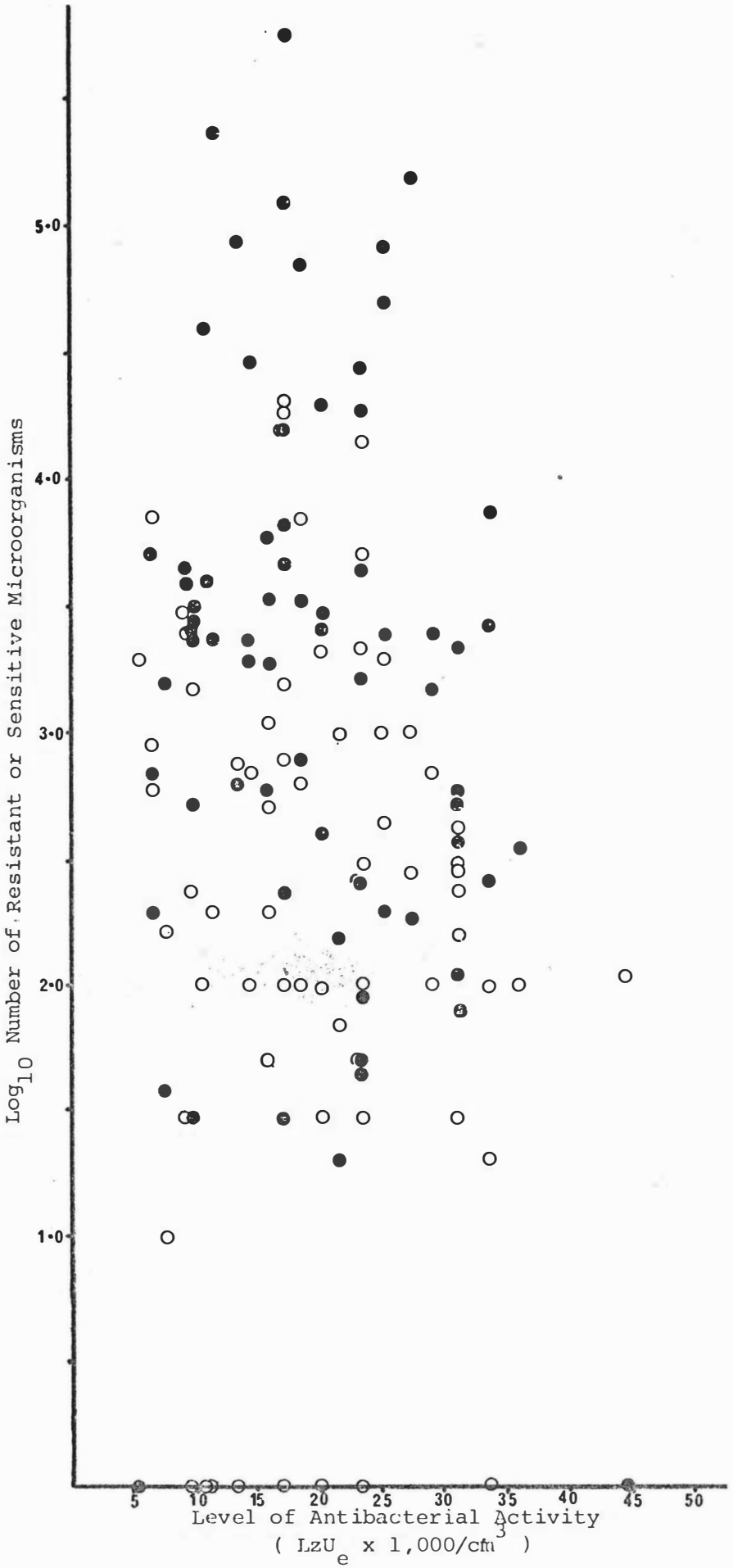


FIGURE FOURTEEN: The Number of Microorganisms Resistant to (●), or Sensitive to (O) Bovine Seminal Plasma Inhibition in Relation to the amount of this Activity in a Given Semen Sample.

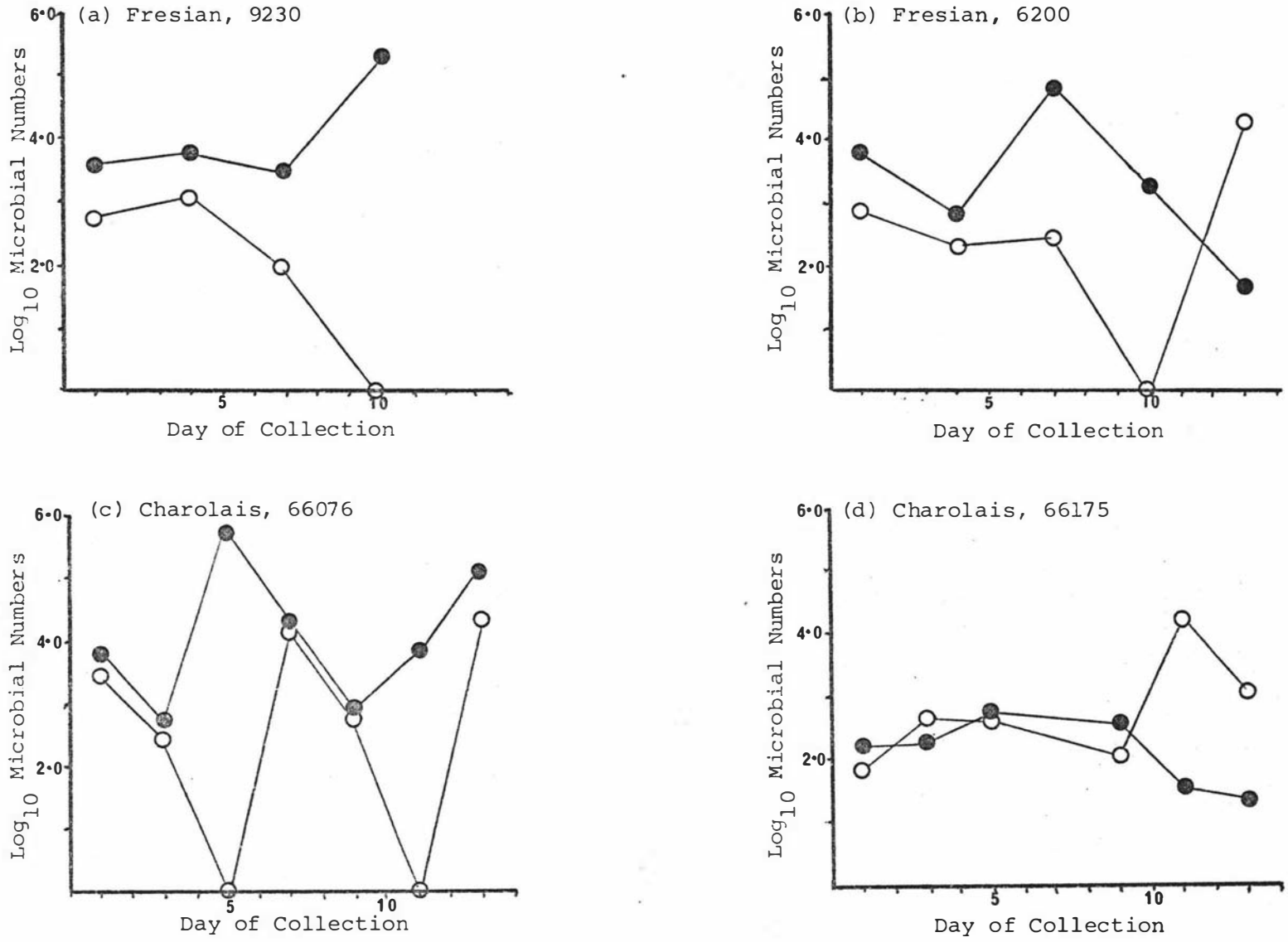


FIGURE FIFTEEN: Day to Day Variations in the Number of Microorganisms Resistant to (●), or Sensitive to (○) the Natural Antibacterial Activity of Bovine Seminal Plasma.

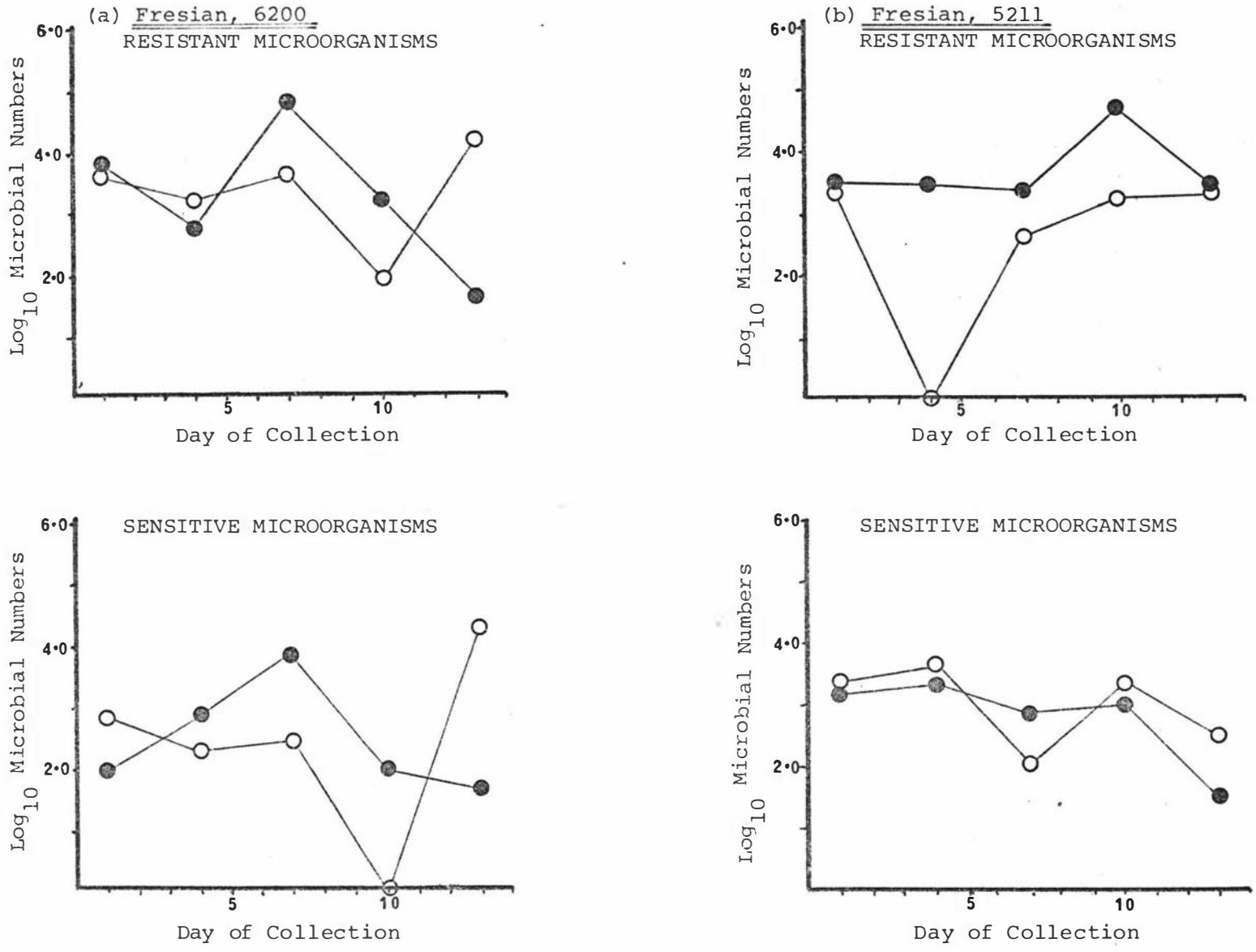


FIGURE SIXTEEN: Day to Day Fluctuations in First (●) and Second (○) Ejaculates of Microbial Populations Resistant to or Sensitive to the Natural Antibacterial Activity of Bovine Seminal Plasma.

statistical significance (using the "t" test, Bailey 1968) between the resistant populations of first (logarithmic average 2.0 x 10^3 organisms /cm^3) and second (logarithmic average 6.1 x 10^2 organisms/cm^3) ejaculates due to wide fluctuations in the populations of both ejaculates, a decrease in the number of resistant organisms from first to second ejaculates occurred in 13/18 (or 72%) of such pairs examined. This may reflect a washing out of temporary invaders of the lower reproductive tract, which are able to persist in this location due to their resistance to bovine seminal plasma inhibition. The second ejaculate would contain fewer such microorganisms.

No large populations of microorganisms sensitive to bovine seminal plasma inhibition occurred in the semen samples examined. The range of sensitive organisms present in semen varied from 0 to 2.0 x 10^4 organisms/ cm^3, with a logarithmic average of 1.9 x 10^2 organisms/cm^3. The number of sensitive organisms did not vary between first (logarithmic average 3.7 x 10^2 colonies/cm^3) and second (logarithmic average 2.3 x 10^2 colonies /cm^3) ejaculates, with equal numbers of pairs showing an increase or a decrease in sensitive populations between the two samples. Examination of the number of morphologically different colonies showed that 44% of the distinguishable colony types present in semen culture were sensitive to the natural antibacterial activity of bovine seminal plasma. It is suggested that these organisms are transient contaminants, probably from the preputial cavity and the urethra. unable to persist in ejaculated semen or the reproductive tract for long periods of time.

12.4 The Effect of the Antibacterial Activity of Bovine Seminal Plasma on Several Genera of Bacteria isolated from Bovine Semen

The standard sensitivity test (CHAPTER 5.3) for bovine seminal plasma inhibitors was adapted to measure the resistance of representative strains of bacteria isolated from semen by substituting the test organism for M. lysodeikticus. The response of these semen micororganisms to the natural antibacterial activity of bovine seminal plasma are recorded in TABLE VII. These results indicate that seminal plasma is effective against 26/38 (or 68%) of the strains tested. The sensitive organisms represent 10 different genera.

Six genera proved to contain bacteria resistant to the natural inhibitors present in bovine semen. Of these, Bacillus and Corynebacterium contained strains some of which were sensitive to seminal plasma inhibition while others were not affected. Gram-negative bacteria seemed

TABLE VII.

THE EFFECT OF THE ANTIBACTERIAL ACTIVITY OF BOVINE SEMINAL PLASMA ON BACTERIA ISOLATED FROM BOVINE SEMEN

GENUS	STRAINS EXAMINED	INHIBITION ZONE DIAMETER (mm)				TOTALS	
		0	9-12	13-17	17 +	resistant	sensitive
Pseudomonas	4	4	0	0	0	4	0
Alcaligenes	1	1	0	0	0	1	0
Proteus	1	1	0	0	0	1	0
Staphylococcus	1	1	0	0	0	1	0
Corynebacterium	7	3	0	3	1	3	4
Bacillus	9	2	0	6	1	2	7
Aerobacter	2	0	0	2	0	0	2
Arthrobacter	1	0	0	0	1	0	1
Brevibacterium	1	0	0	0	1	0	1
Escherichia	2	0	0	2	0	0	2
Microbacterium	1	0	0	1	0	0	1
Micrococcus	6	0	1	3	2	0	6
Nocardia	1	0	0	0	1	0	1
Sarcina*	1	0	0	1	0	0	1
TOTAL	38	12	1	18	7	12	26

FOOTNOTE: * currently classified as Micrococcus (Bergey 1974)

more likely to be resistant than Gram-positive organisms, since of all the former strains, 6/10 (or 60%) were resistant, compared with 6/28 (or 21%) Gram-positive bacteria. All strains (4) of Pseudomonas tested were resistant to the antibacterial activity of bovine seminal plasma, but the 4 strains of Aerobacter and Escherichia considered here were sensitive to the natural antibacterial agents in bovine semen, although resistant to many of the commercially available antibiotics (APPENDIX FOUR).

RESULTS : PART TWO

THE NATURE OF THE ANTIMICROBIAL ACTIVITY IN BOVINE SEMEN AND BOVINE SEMINAL PLASMA

The chemical and physical characterisation of inhibitory factors present in bovine seminal plasma was undertaken using pooled vasectomised bovine seminal plasma, and selected antimicrobial agents were studied for comparison.

CHAPTER 13: THE CHEMICAL CHARACTERISATION OF THE ANTIBACTERIAL ACTIVITY IN BOVINE SEMINAL PLASMA.

13.1 The Sensitivity of Bovine Seminal Plasma Antibacterial Activity to Proteolytic Enzymes, and a Comparison with the Activities of Selected Antimicrobial Agents.

The antibacterial activity of bovine seminal plasma proved sensitive to all three proteolytic enzymes tested (TABLE VIII). Subtilisin (causing nonspecific peptide bond hydrolysis) and thermolysin (attacking leucyl / phenylalanyl residues) completely destroyed its antibacterial activity, whereas trypsin (attacking lysyl / arginyl residues) resulted in a 95 to 99% loss in measurable activity (CHAPTER 7.2).

When the effect of trypsin treatment on bovine seminal plasma inhibitory activity was compared with its effect on selected antimicrobial agents, lysozyme, polylysine and salmine were all found to be sensitive to trypsin, losing 100, 98, and 99% activity respectively. Trypsin had no effect on the antibacterial activity of spermidine (TABLE IX).

Using Staphylococcus aureus as the indicator organism, the antibacterial activity of bovine seminal plasma was lost from all samples (with and without proteolytic enzymes) due to the holding temperature in the experimental procedure (CHAPTER 7.2). Lysozyme had no antibacterial activity against this bacterium. Trypsin completely destroyed the antibacterial activity of polylysine and salmine. Spermidine activity was again unaffected by trypsin treatment.

13.2 The Effect of Periodate on the Antibacterial Activity of Bovine Seminal Plasma and those of Selected Antimicrobial Agents.

Bovine seminal plasma, lysozyme, polylysine and salmine showed no decrease in antibacterial activity against M. lysodeikticus after treatment with periodate (CHAPTER 7.3). The antibacterial activity of spermidine was, however, completely destroyed by this treatment (TABLE X).

TABLE VIII.

THE EFFECT OF PROTEOLYTIC ENZYMES ON THE ANTIBACTERIAL ACTIVITY OF BOVINE SEMINAL PLASMA

ENZYME	BOVINE SEMINAL PLASMA ACTIVITY[1]		PERCENT DECREASE IN ACTIVITY AFTER TREATMENT
	before treatment	after treatment	
Thermolysin	62,000	0	100
Subtilisin	62,000	0	100
Trypsin	62,000	820	99

FOOTNOTE: [1] Activity expressed as lysozyme unit equivalents (LzU_e), see CHAPTER 4.4 .

TABLE IX.

THE EFFECT OF TRYPSIN TREATMENT ON THE ANTIBACTERIAL ACTIVITY OF BOVINE SEMINAL PLASMA AND SELECTED ANTIMICROBIAL AGENTS.

TEST SOLUTION	ANTIBACTERIAL ACTIVITY *				PERCENT DECREASE IN ACTIVITY AFTER TREATMENT	
	before treatment		after treatment			
	M[1]	S[2]	M[1]	S[2]	M[1]	S[2]
Bovine seminal plasma	62,500	0	2,900	0	95	-
Lysozyme	700,000	0	410	0	100	-
Polylysine	280,000	9,000	4,400	0	98	100
Salmine	102,500	6,400	940	0	99	100
Spermidine	360	36,000	360	36,000	0	0

FOOTNOTES: * Activity expressed as lysozyme unit equivalents (LzU_e), see CHAPTER 4.4 .

[1] M = using Micrococcus lysodeikticus as the indicator organism in the antibacterial assay.

[2] S = using Staphylococcus aureus as the indicator organism in the antibacterial assay.

TABLE X.

THE EFFECT OF PERIODATE TREATMENT ON THE ANTIBACTERIAL ACTIVITY OF BOVINE SEMINAL PLASMA AND SELECTED ANTIMICROBIAL AGENTS.

TEST SOLUTION	ANTIBACTERIAL ACTIVITY* before treatment		after treatment		PERCENT DECREASE IN ACTIVITY AFTER TREATMENT	
	M[1]	S[2]	M[1]	S[2]	M[1]	S[2]
Bovine seminal plasma	47,500	0	47,500	0	0	-
Lysozyme	430,000	0	430,000	0	0	-
Polylysine	150,000	9,600	150,000	9,600	0	0
Salmine	81,000	4,800	81,000	4,800	0	0
Spermidine	360	23,250	0	0	100	100

FOOTNOTES: * Activity expressed as lysozyme unit equivalents (LzU_e), see CHAPTER 4.4 .

[1] M = using Micrococcus lysodeikticus as the indicator organism in the antibacterial assay.

[2] S = using Staphylococcus aureus as the indicator organism in the antibacterial assay.

TABLE XI.

THE EFFECT OF HEAT (60'C for 60 minutes) ON THE ANTIBACTERIAL ACTIVITY OF BOVINE SEMINAL PLASMA AND SELECTED ANTIMICROBIAL AGENTS.

TEST SOLUTION	ANTIBACTERIAL ACTIVITY* before treatment		after treatment		PERCENT DECREASE IN ACTIVITY DUE TO HEATING.	
	M[1]	S[2]	M[1]	S[2]	M[1]	S[2]
Bovine seminal plasma	62,500	360	53,800	0	14	100
Lysozyme	900,000	0	900,000	0	0	-
Polylysine	430,000	10,500	185,000	12,250	57	0
Salmine	200,000	7,000	78,000	7,000	61	0
Spermidine	2,900	50,000	2,560	75,000	12	0

FOOTNOTES: * Activity expressed as lysozyme unit equivalents (LzU_e), see CHAPTER 4.4 .

[1] M = using Micrococcus lysodeikticus as the indicator organism in the antibacterial assay.

[2] S = using Staphylococcus aureus as the indicator organism in the antibacterial assay.

13.3 The Heat Sensitivity of Bovine Seminal Plasma Antimicrobial Activity.

Bovine seminal plasma held at 60, 70 and 80'C for 60 minutes still retained some antibacterial activity. A constant proportion (approximately 36%) of the overall activity appeared to be heat stable even at 80'C (FIGURE SEVENTEEN).

Bovine seminal plasma loses little (up to 14%) or none of its overall antibacterial activity at 60'C for 60 minutes. Under these conditions, lysozyme, polylysine, salmine and spermidine lose 0, 57, 61 and 12% of their inhibitory activity respectively (TABLE XI). The anti-activity of polylysine, salmine and spermidine against Staphylococcus aureus was not affected by heating to 60'C for 60 minutes. Lysozyme did not inhibit S. aureus .

During these heat sensitivity studies it was noted that there was some indication that the antimicrobial activity of bovine seminal plasma may be associated with more than one individual factor. Untreated seminal plasma was active against a number of bacteria including Micrococcus lysodeikticus, Escherichia coli, a strain of Staphylococcus aureus, a strain of Bacillus subtilis and an unidentified bacillus species, used by Shannon et al (1974) as their indicator organism. Bovine seminal plasma was also active against several Mycoplasma species, including M. hominis, M. arthritidis and M. ovipneumoniae (CHAPTER 16.2,see TABLE XVIII). Bovine seminal plasma which was heated to 60'C for 60 minutes was inactive against the B. subtilis strain and a strain of S. aureus, whereas its activity against M. lysodeikticus, E. coli, the Bacillus species and and Mycoplasmas was undiminished (TABLE XII).

The possibility that a labile antibacterial fraction is present in bovine seminal plasma and is responsible for some of its activity was comfirmed by the following experiment. The antibacterial activity of two different batches of pooled seminal plasma against both M. lysodeikticus and the S. aureus strain were constant after storage at -20'C for six months. Heating aliquots of this stored material to 60'C for 60 minutes had no effect on its activity against M. lysodeikticus but completely destroyed its activity against the S. aureus strain. " Ageing " the seminal plasma by allowing it to stand at 30'C for 60 minutes also had no effect on its activity against M. lysodeikticus but against the S. aureus strain, the seminal plasma samples lost 14% and 100% of their activity respectively, after the the 30'C treatment (TABLE XIII). These results suggest that there is an extremely labile compound or complex

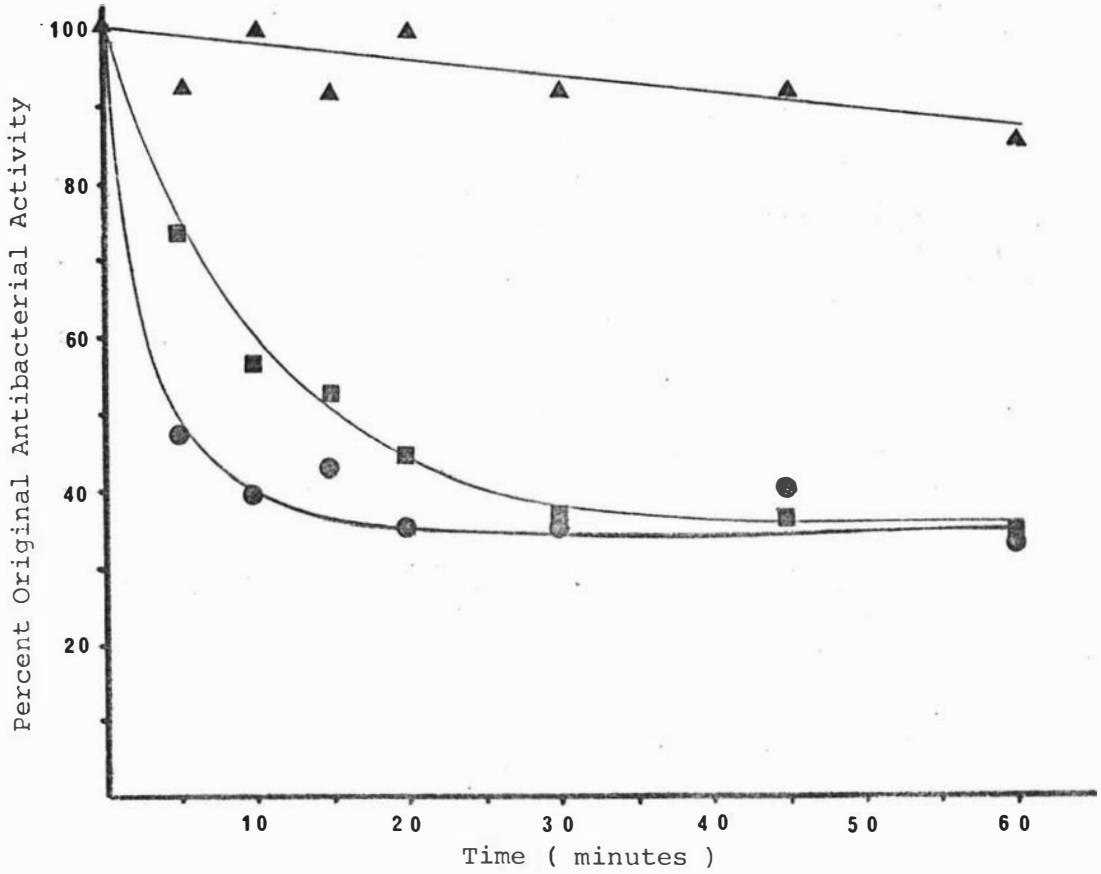


FIGURE SEVENTEEN: The Heat Sensitivity of the Antibacterial Activity of Bovine Seminal Plasma, assayed against Micrococcus lysodeikticus.

▲ = 60'C
■ = 70'C
● = 80'C

TABLE XII.

THE EFFECT OF HEATING BOVINE SEMINAL PLASMA TO 60'C for 60 MINUTES ON ITS ANTIMICROBIAL ACTIVITY.

TEST ORGANISM	BOVINE SEMINAL PLASMA ACTIVITY*		PERCENT DECREASE IN ACTIVITY DUE TO HEATING
	before heat	after heat	
Micrococcus lysodeikticus	76,000	76,000	0
Escherichia coli	9,000	9,000	0
Proteus vulgaris	0	0	-
Staphylococcus aureus	720	0	100
Staphylococcus aureus	14,250	0	100
Bacillus subtilis	1,325	0	100
Bacillus sp.[1]	11,250	11,250	0
Mycoplasma hominis	740,000	540,000	27
Mycoplasma arthritidis	340,000	280,000	18
Mycoplasma ovipneumoniae	102,500	81,000	21

FOOTNOTES: * Activity expressed as lysozyme unit equivalents (LzU_e), see CHAPTER 4.4 .

[1] Isolated by Shannon et al (1974).

TABLE XIII.

THE EFFECT OF " AGEING " (30'C for 60 minutes) ON THE ANTIBACTERIAL ACTIVITY OF BOVINE SEMINAL PLASMA.

BOVINE SEMINAL PLASMA	ANTIBACTERIAL ACTIVITY*		PERCENT DECREASE IN ACTIVITY	
	M[1]	S[2]	M[1]	S[2]
Sample 1,untreated	31,000	550		
" ,30'C for 60 minutes	31,000	0	0	100
" ,60'C for 60 minutes	31,000	0	0	100
Sample 2,untreated	20,000	360		
" ,30'C for 60 minutes	20,000	310	0	14
" ,60'C for 60 minutes	20,000	0	0	100

FOOTNOTES: * Activity expressed as lysozyme unit equivalents (LzU_e), see CHAPTER 4.4 .

[1] M = using Micrococcus lysodeikticus as the indicator organism in the antibacterial assay.

[2] S = using Staphylococcus aureus as the indicator organism in the antibacterial assay.

associated with the inhibitory effects of bovine seminal plasma against the S. aureus strain. The level of activity and its reaction to " ageing " appears to vary in different samples.

CHAPTER 14: THE EFFECT OF SODIUM CITRATE ON THE ANTIBACTERIAL ACTIVITY OF BOVINE SEMINAL PLASMA.

14.1 The Effect of Varying Amounts of Citrate on the Inhibition Zones Produced by Bovine Seminal Plasma Antibacterial Activity.

When sodium citrate was used to elute the active fraction of bovine seminal plasma off deoxyribonucleic acid (DNA) in an extraction process (CHAPTER 19.4), it was noted that the inhibition zones produced by seminal plasma containing citrate were larger than those of non - citrated samples. When this was investigated, it was found that the presence of sodium citrate in bovine seminal plasma samples increased the diameter of the inhibition zones produced in the standard agar well diffusion assay (CHAPTER 4.3). Samples which contained more than 0.5M citrate formed large diffuse zones of inhibition. A 0.5M citrate solution produced a similar effect. At sample levels of less than 0.5M, however, citrate increased the diameter of the inhibition zones produced by bovine seminal plasma in a uniform manner (FIGURE EIGHTEEN).

A similar but lesser effect was seen with the presence of varying levels of sodium chloride in the sample. The presence of NaCl also resulted in some enhancement of the diameter of the inhibition zone produced by partially purified (acetone precipitated,pH2.5 acid dialysate of bovine seminal plasma,see CHAPTER 18.1) antimicrobial material (FIGURE NINETEEN).

14.2 Modification of the Agar Well Diffusion Assay to Incorporate Citrate.

It was thought that the larger zones produced in the presence of citrate were due to the dissociation of high molecular weight complexes into smaller moieties which could diffuse further and more rapidly in the agar during the assay. The effect of citrate incorporated into the agar on the diameter of the inhibition zones was examined. Sodium citrate can be incorporated into the assay agar up to a concentration of 0.1M, higher levels interfered with the growth of the indicator organism. From FIGURE EIGHTEEN, it can be seen that 0.1M citrate in the agar resulted in greater variability and smaller inhibition zones produced by seminal plasma samples containing varying levels of citrate than was the case with 0.05M citrate incorporated into the agar. The greatest sensitivity and least variation in the diameter of the inhibition zone for a given bovine seminal plasma sample, regardless of the level of citrate present in the sample, was obtained with 0.05M citrate present in the assay agar.

Closer inspection of the effect of citrate levels produced the

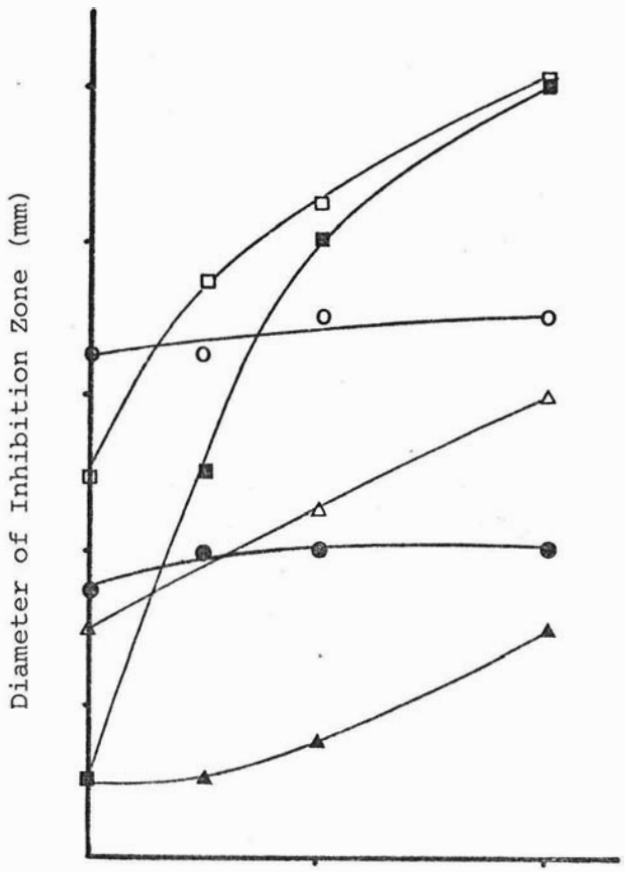


FIGURE EIGHTEEN: The Effect of Varying Amounts of Citrate in Bovine Seminal Plasma on the Inhibition Zones produced in radial diffusion Assays.

□= sample 1, 0.00M citrate in assay agar
o= " , 0.05M " " " "
△= " , 0.10M " " " "

■= sample 2, 0.00M citrate in assay agar
●= " , 0.05M " " " "
▲= " , 0.10M " " " "

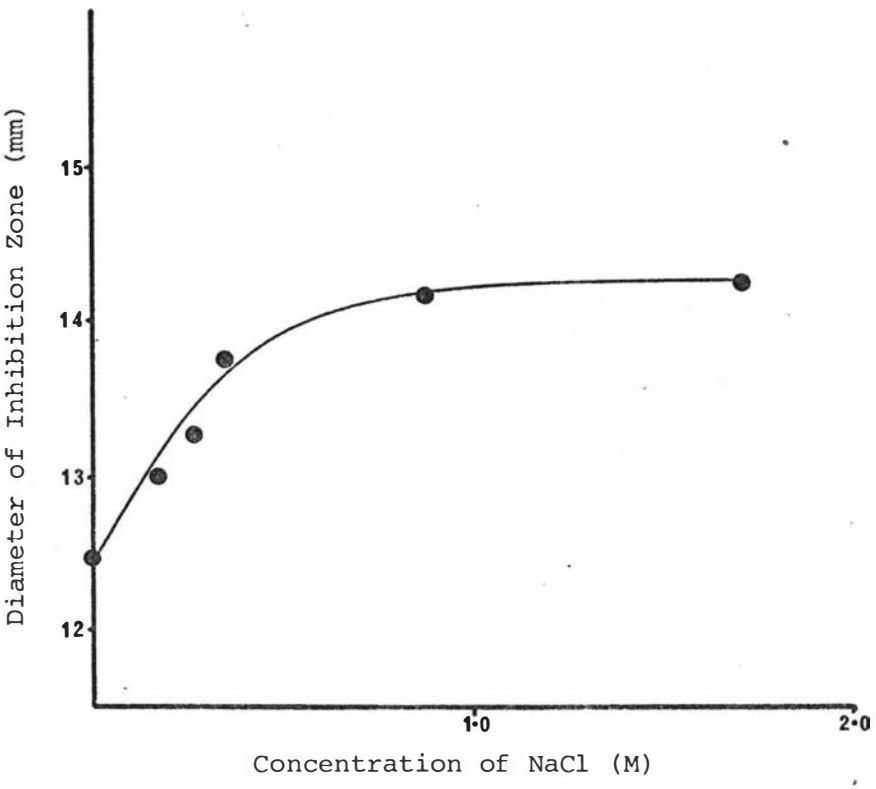


FIGURE NINETEEN: The Effect of Varying Amounts of NaCl in Partially Purified (acetone precipitated acid dialysate, CHAPTER 18.1) Antimicrobial Material (5mg/cm^3) on the Inhibition Zones Produced in Radial Diffusion Assays.

results shown in FIGURE TWENTY . Thus agar levels of 0.0225M citrate gave very consistent measures of zone diameter regardless of the amount of citrate present in the sample. In addition FIGURE TWENTY ONE shows that at this level, the assay is at its greatest sensitivity. A $10mg/cm^3$ solution of partially purified antimicrobial material (acetone precipitated,pH2.5 acid dialysate of bovine seminal plasma, CHAPTER 18.1), with 0 to 0.3M sodium citrate gave a consistant reading of 20.5mm diameter of inhibition zone with 0.0225M citrate in the assay agar. Thus the activity of samples with citrate levels up to 0.3M can be successfully and accurately measured using citrated nutrient agar as the growth medium for the agar well diffusion assay. This level of citrate was routinely used for all assays of antibacterial activity (CHAPTER 4.4).

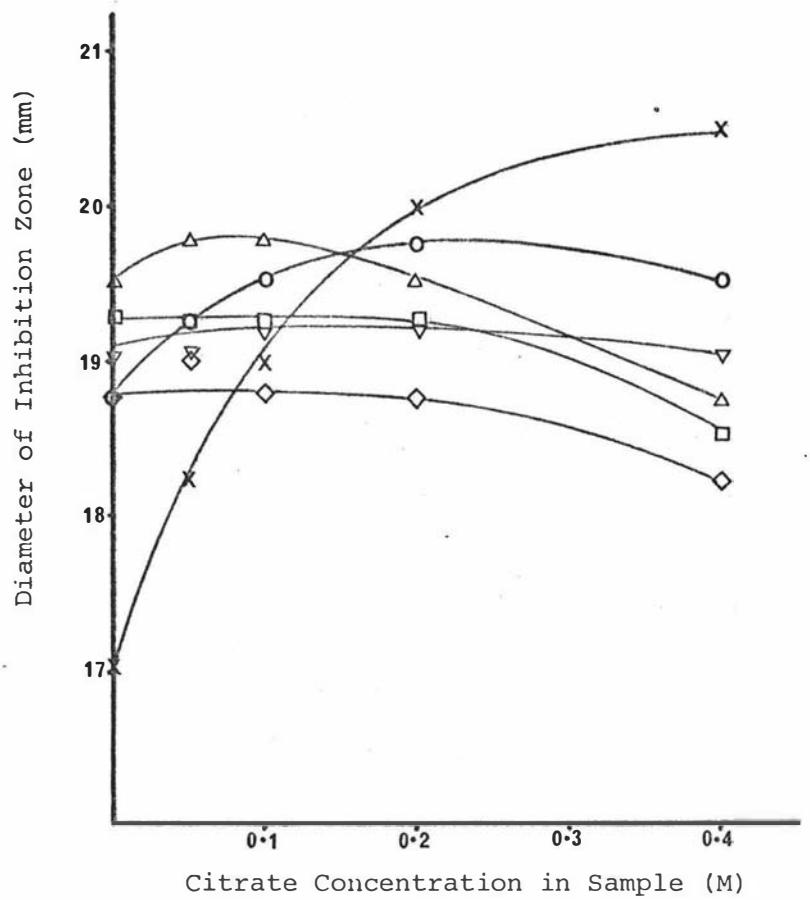


FIGURE TWENTY: The Effect of Citrate Incorporated into the Assay Agar on the Diameter of the Inhibition Zone produced by Bovine Seminal Plasma containing Varying Amounts of Citrate.

X = 0.0000M citrate in the assay agar
O = 0.0100M " " " " "
△ = 0.0200M " " " " "
▽ = 0.0225M " " " " "
□ = 0.0250M " " " " "
◇ = 0.0300M " " " " "

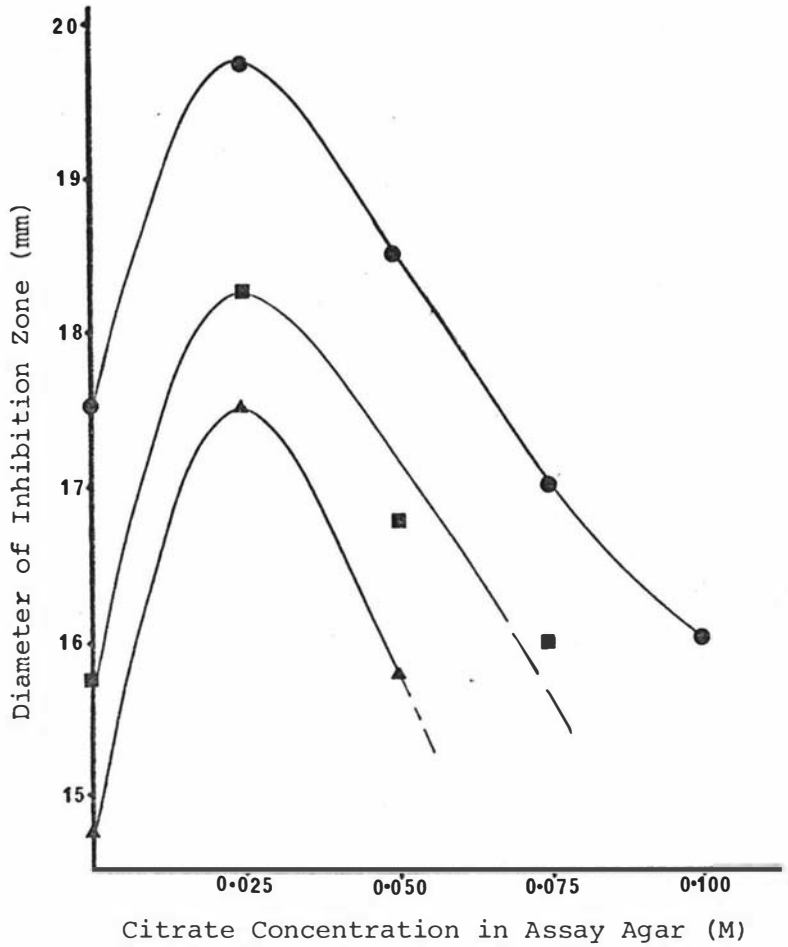


FIGURE TWENTY ONE: The Sensitivity of Assays using Varying Amounts of Citrate incorporated into the Agar to Bovine Seminal Plasma without added Citrate.

●= Bovine seminal plasma sample 1
■= " " " " 2
▲= " " " " 3

CHAPTER 15: INVESTIGATION OF THE PHYSICAL STATE OF THE ANTIMICROBIAL FACTORS IN BOVINE SEMINAL PLASMA.

The physical state of the factors responsible for the antimicrobial activity of bovine seminal plasma was studied by examining the effect of dialysis, ultrafiltration and native polyacrylamide gel electrophoresis on this activity.

15.1 The Absorbance Spectrum of Bovine Seminal Plasma.

The absorbance of bovine seminal plasma and a crude preparation of its antimicrobial activity (acetone precipitated, pH2.5 acid dialysate, CHAPTER 18.1) , was measured over wavelengths 250 to 450nm in a Unicam SP800 spectrophotometer. The major absorption peak occurred at approximately 280nm, with a slight shoulder appearing at 286 to 290nm (FIGURE TWENTY TWO).

15.2 Dialysis of Bovine Seminal Plasma.

Dialysis of bovine seminal plasma adjusted to pH 2.5 with hydrochloric acid against an equal volume of pH 2.5 acid for three days at 4'C, resulted in 14% of its overall activity diffusing through the membrane. Further dialysis of the material within the dialysis sac against fresh acid for an additional three days resulted in the loss of another 7% of the original activity into the dialysing fluid (TABLE XIV).

15.3 Ultrafiltration of Bovine Seminal Plasma.

When bovine seminal plasma was reduced to 1/5th its original volume using an XM 50 ultrafiltration membrane in the Amicon unit (CHAPTER 7.4), 33% of the overall antibacterial activity was present in the filtrate. Washing the solution retained by the membrane with an equal volume of distilled water and then reducing its volume to 1/5 again, yielded a further 5.6% activity in the filtrate. Thus 61.4% of the total activity in bovine seminal plasma was retained by a membrane which retains molecules of more than 50,000 molecular weight (TABLE XV).

Refiltration of the material which had passed through the XM 50 membrane gave a result which provided additional information ; 24% of the material which had previously passed through the ultrafiltration membrane was now retained by the membrane when the washing process described above was repeated. There was no measurable antibacterial activity in the wash fluid.

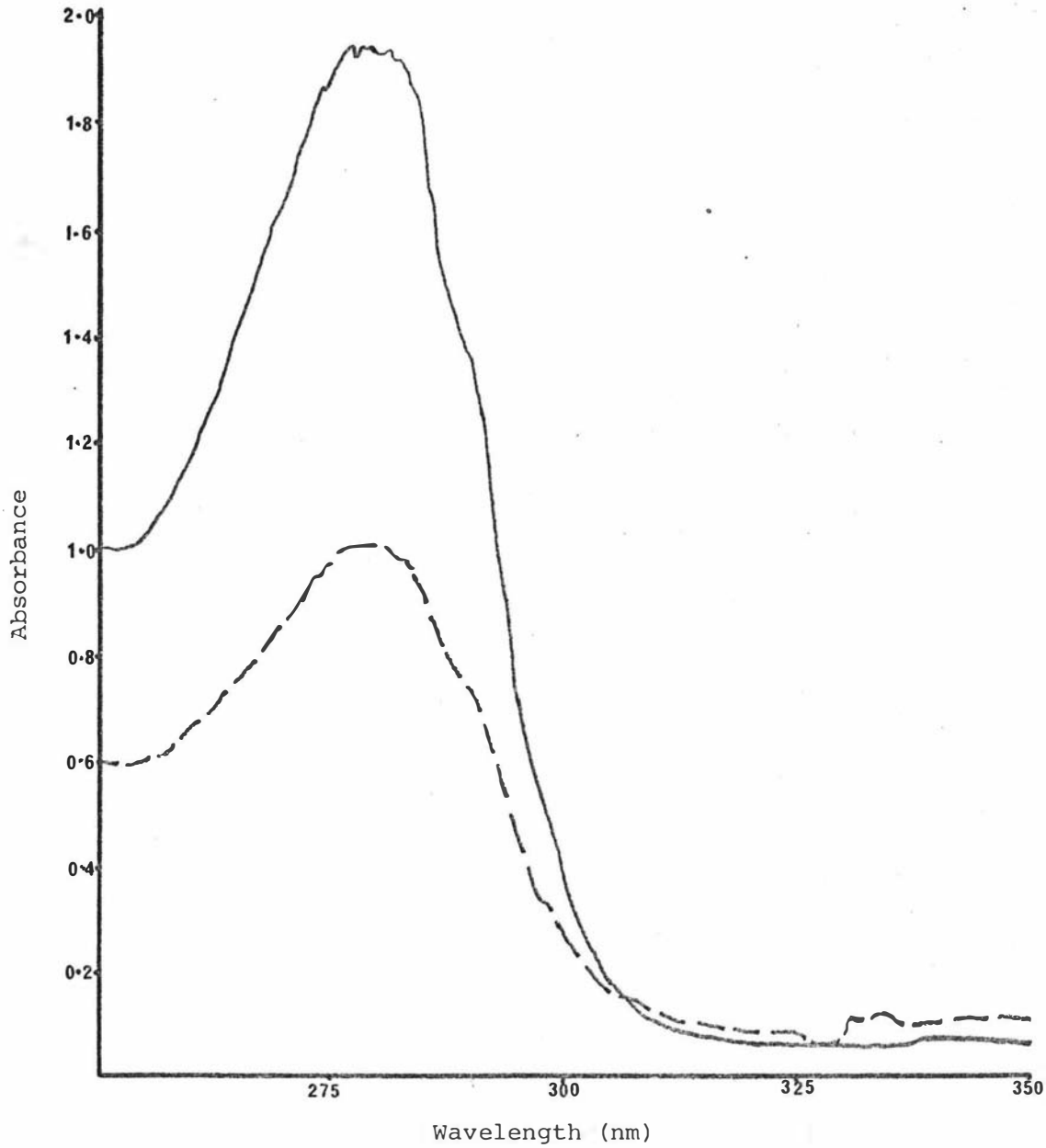


FIGURE TWENTY TWO: Absorbance Spectra of Bovine Seminal Plasma (— ~ —, 0.5cm^3/cm^3 pH 8.6 Tris/Glycine buffer), and Partially Purified Material (~~~, acetone precipitated acid dialysate 10mg/cm^3 pH 8.6 Tris/Glycine buffer).

TABLE XIV.

THE EFFECT OF DIALYSIS ON THE ANTIBACTERIAL ACTIVITY OF BOVINE SEMINAL PLASMA.

TEST SOLUTION	TOTAL ACTIVITY*	PERCENT ORIGINAL ACTIVITY
Untreated bovine seminal plasma	32,000	100.0
First dialysate	4,450	13.9
Second dialysate	2,100	6.6

FOOTNOTE : * Activity expressed in lysozyme unit equivalents (LzU_e),see CHAPTER 4.4 .

TABLE XV.

THE EFFECT OF ULTRAFILTRATION ON THE ANTIBACTERIAL ACTIVITY OF BOVINE SEMINAL PLASMA

TEST SOLUTION	TOTAL ACTIVITY*	PERCENT ORIGINAL ACTIVITY
Filtrate ($20cm^3$)	97,500	33.0
Wash ($20cm^3$)	16,500	5.6
Solution behind membrane ($5cm^3$)	181,250	61.4
Filtrate from refiltration ($12cm^3$)	17,580	24.1
Wash ($12cm^3$)	0	0.0
Solution behind membrane ($3cm^3$)	55,410	75.9

FOOTNOTE: * Activity expressed as lysozyme unit equivalents (LzU_e), see CHAPTER 4.4 .

15.4 Native Polyacrylamide Gel Electrophoresis of Bovine Seminal Plasma.

The method described in CHAPTER 7.5, was used to compare the proteins in bovine seminal plasma with those precipitated by acetone from a dialysate of seminal plasma at pH 2.5 (CHAPTER 18.1) , and with lysozyme. FIGURE TWENTY THREE shows densitometer tracings from a slab gel showing cationic migration. Bovine seminal plasma contained many cationic proteins, among which four major peaks were detected. The extract contained two major peaks both of which were distinct from the major peaks in whole seminal plasma. Lysozyme peaks did not correspond with those in either of the other samples examined.

The electrophoretic properties of the acetone precipitated material CHAPTER 18.1) were also examined at three other pHs. These were :
pH 3.0 using a 0.2M glycine / HCl buffer
pH 5.6 using an acetate / acetic acid buffer and
pH 7.3 using a Tris / HCl buffer.
These were in addition to the pH 8.6 Tris / glycine buffer routinely used. In this study,one section of the completed gel was stained for protein bands (CHAPTER 7.5) and the other section was examined for antibacterial activity using the overlay technique described in CHAPTER 7.6 . The results of this protein staining and biological assay are shown in FIGURE TWENTY FOUR (a) and (b) and FIGURE TWENTY FIVE (a) and (b). At the lower pHs, there was little protein migration during electrophoresis,and the overlay assay located the antibacterial activity very close to the origin. With increasing pH, however, the proteins migrated increasing distances from the origin, and several distinguishable bands occurred, migrating towards both the cathode and the anode. The increasing numbers of anionic proteins were the result of the higher pH being on the alkaline side of their iso-electric points and hence producing molecules with an overall negative charge. Four separate cationic bands were distinguishable at pH 8.6 representing protein molecules with iso-electric points above this pH. Antibacterial activity was found to be associated with at least two of these cationic bands. The activity associated with the largest protein band,which migrated third fastest from the origin,was in greatest concentration, since dilution of the protein sample resulted in the fading of the faster moving antibacterial activity, although the slower moving activity was still present (PLATE ONE).

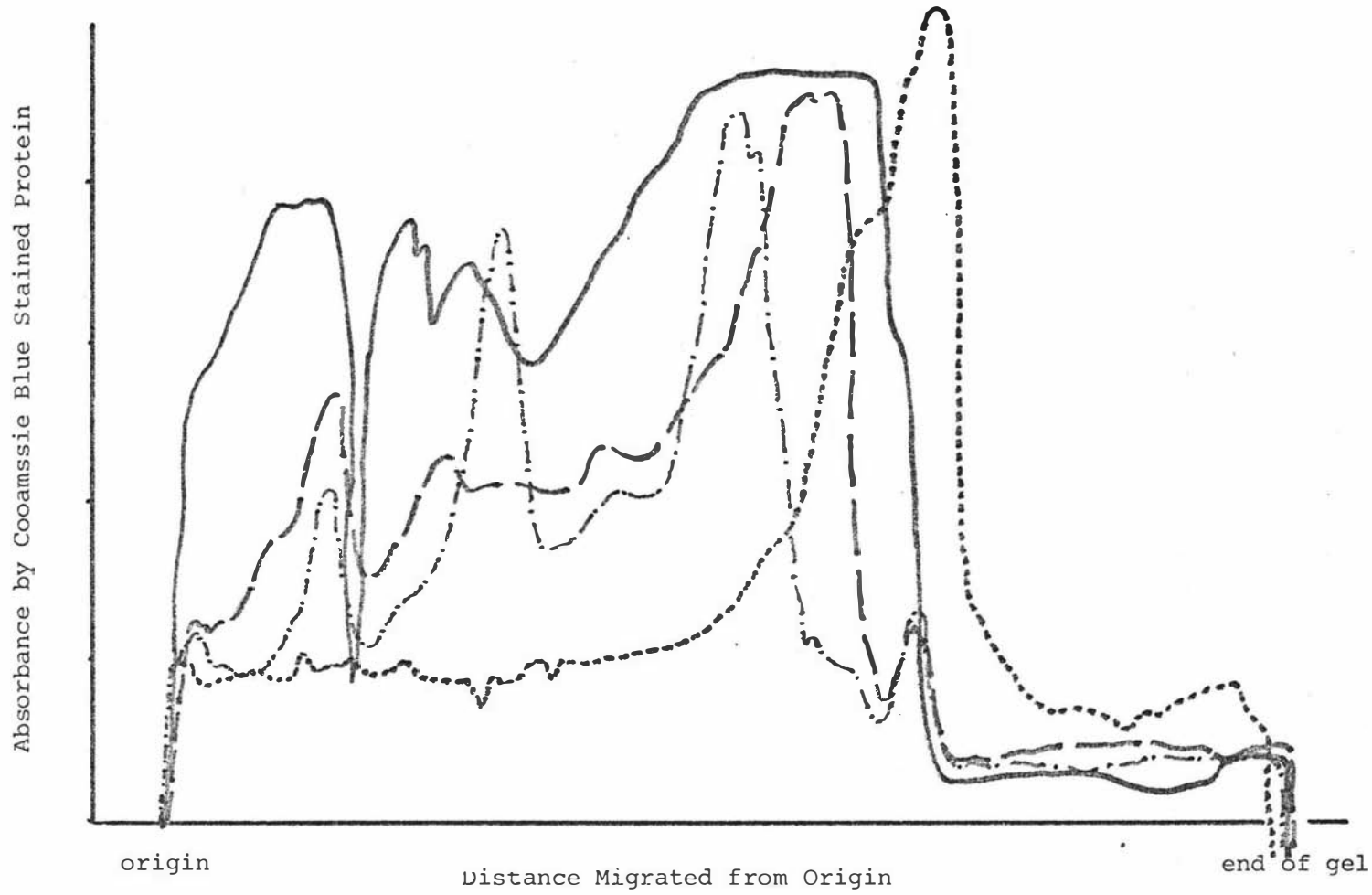


FIGURE TWENTY THREE: Densitometer Tracings of a Slab Gel from Native Polyacrylamide Gel Electrophoresis (pH8.6) of Bovine Seminal Plasma (= diluted 1:1 with distilled water; = diluted 1:3 with distilled water), Partially Purified (CHAPTER 18.1) Antimicrobial Material (= 10mg/cm^3) , and Egg White Lysozyme (= 2.5mg/cm^3).

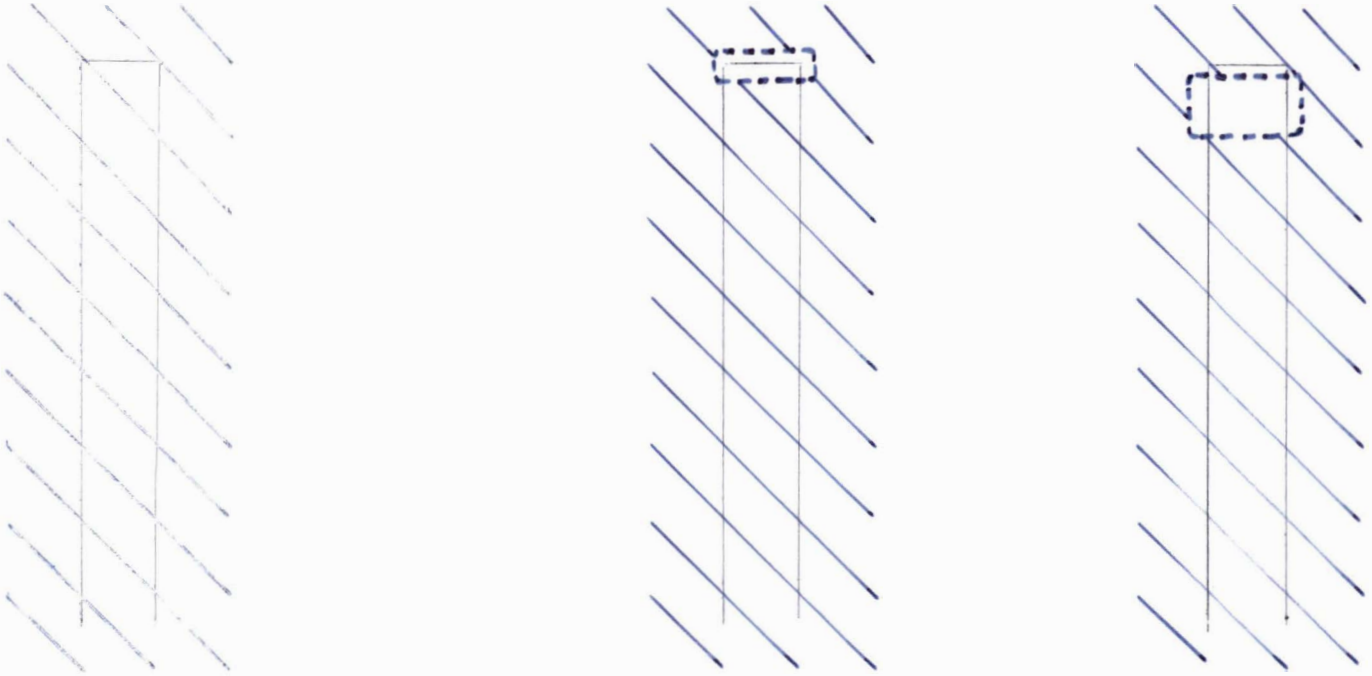


FIGURE TWENTY FOUR (a): Inhibition Zones ( ) produced by Electrophoresed Bovine Seminal Plasma Proteins (1% w/v acetone precipitated acid dialysate, see CHAPTER 18.1) after the Native Polyacrylamide Gels (pH 3.0 and pH 5.6) were overlaid with agar seeded with Micrococcus lysodeikticus.
Low pH affected the growth of the test organism.

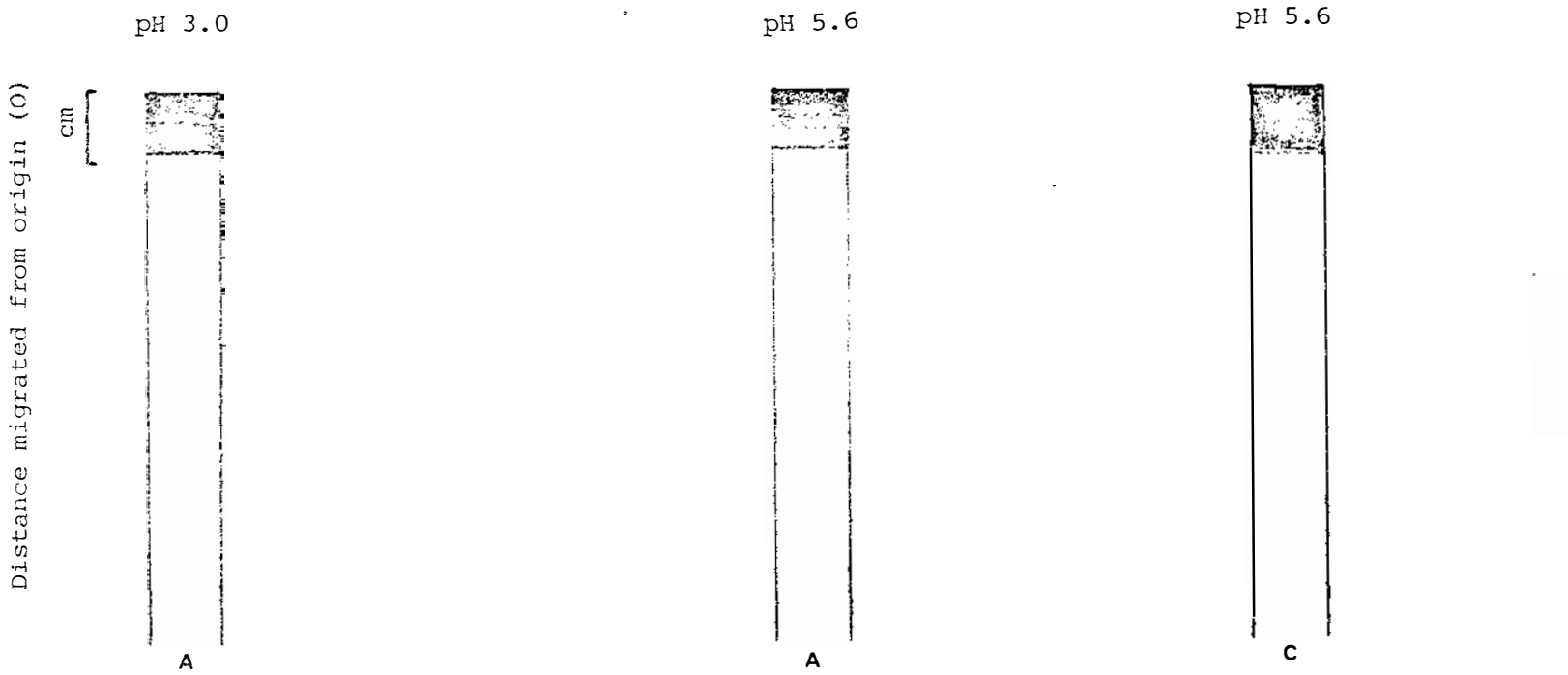


FIGURE TWENTY FOUR (b): Electrophoretic Characteristics (Native Polyacrylamide Gel Electrophoresis, pH 3.0 and pH 5.6) of Bovine Seminal Plasma Proteins (1% w/v acetone precipitated acid dialysate, see CHAPTER 18.1).

A = Anionic Proteins

C = Cationic Proteins

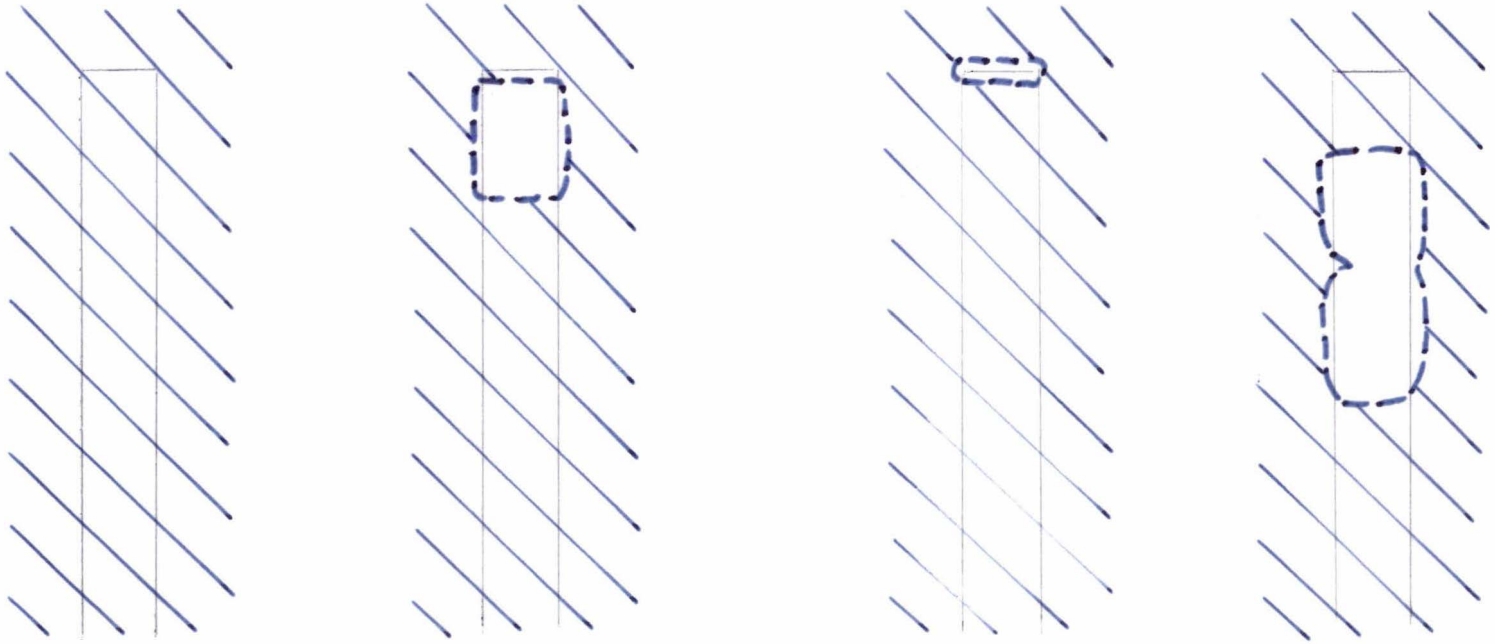


FIGURE TWENTY FIVE (a): Inhibition Zones ( ) produced by Electrophoresed Bovine Seminal Plasma Proteins (1% w/v acetone precipitated acid dialysate, see CHAPTER 18.1) after the Native Polyacrylamide Gels (pH 7.3 and pH 8.6) were overlaid with agar seeded with Micrococcus lysodeikticus.

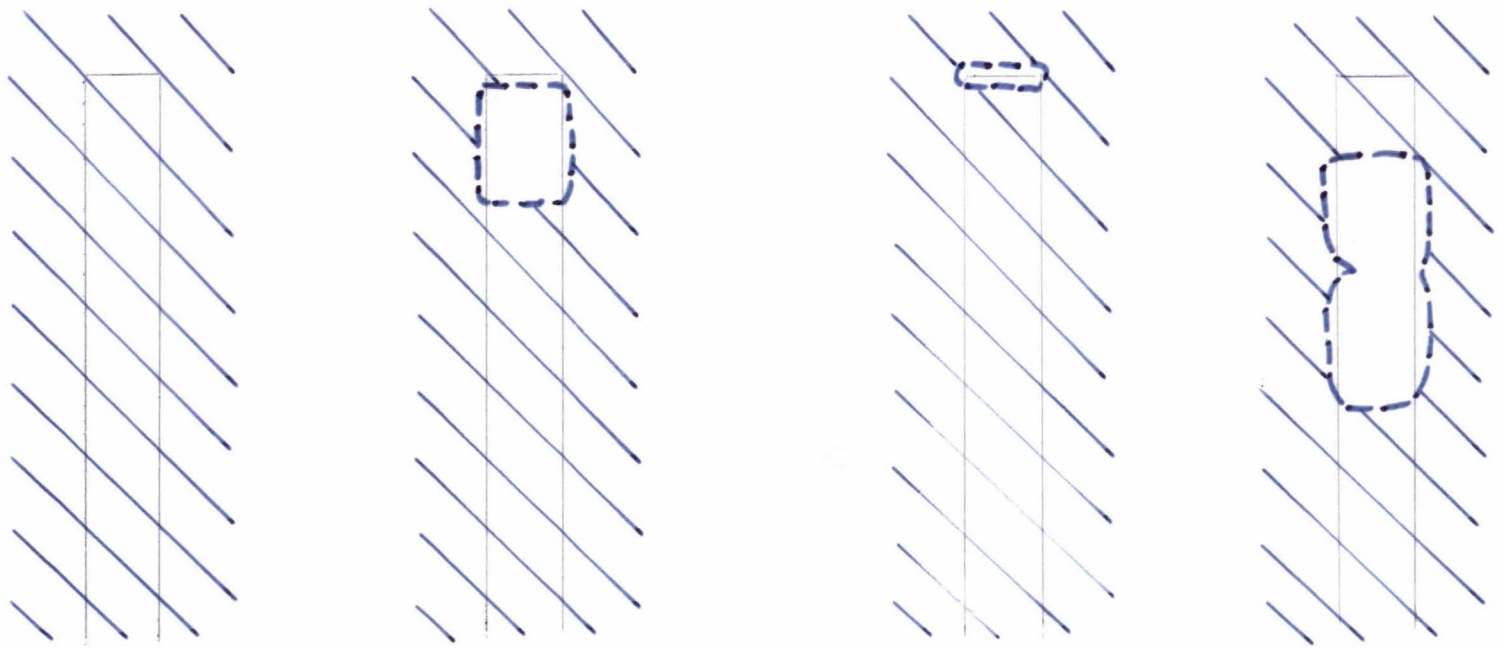


FIGURE TWENTY FIVE (a): Inhibition Zones ( ) produced by Electrophoresed Bovine Seminal Plasma Proteins (1% w/v acetone precipitated acid dialysate,see CHAPTER 18.1) after the Native Polyacrylamide Gels (pH 7.3 and pH 8.6) were overlaid with agar seeded with Micrococcus lysodeikticus.

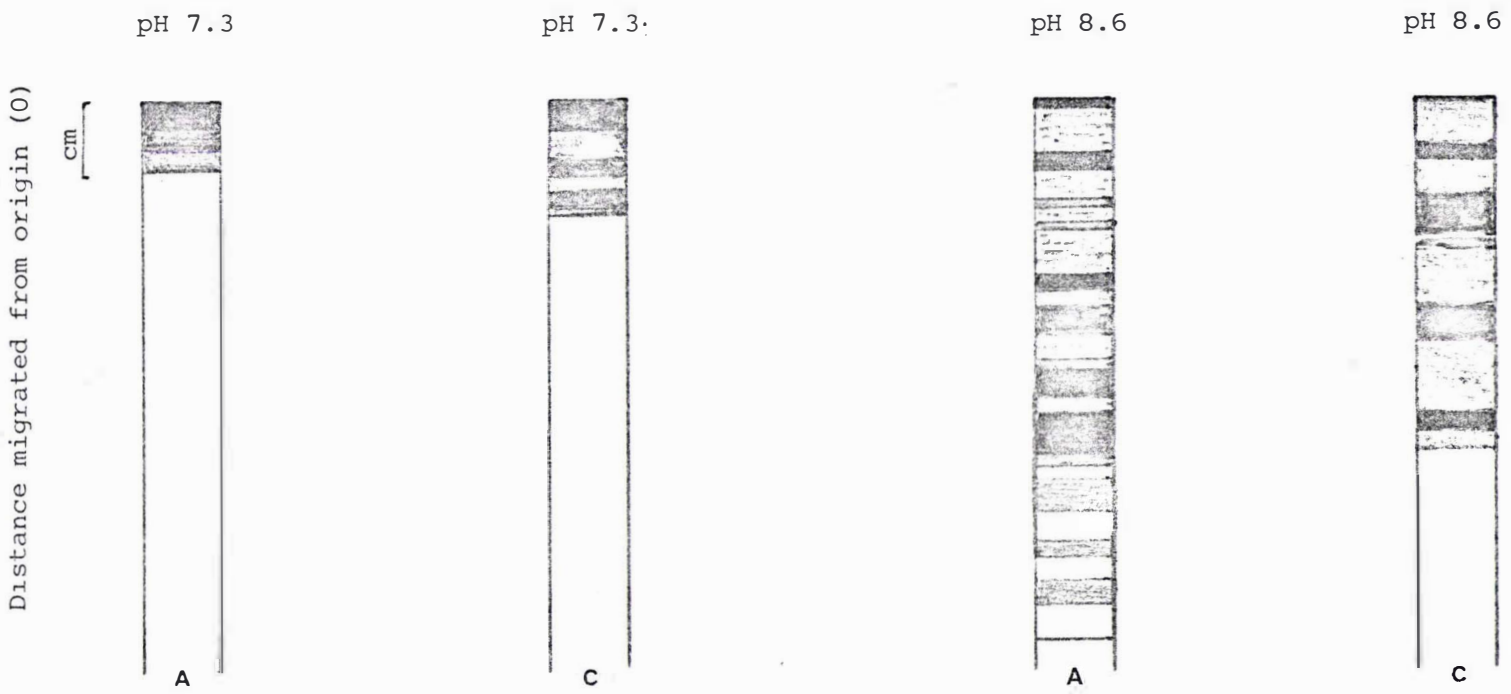


FIGURE TWENTY FIVE (b): Electrophoretic Characteristics (Native Polyacrylamide Gel Electrophoresis, pH 7.3 and pH 8.6) of Bovine Seminal Plasma Proteins (1% w/v acetone precipitated acid dialysate, see CHAPTER 18.1).

A = Anionic Proteins

C = Cationic Proteins

RESULTS : PART THREE

THE SPECTRUM OF ACTIVITY AND MODE OF ACTION OF THE ANTIMICROBIAL ACTIVITY OF BOVINE SEMINAL PLASMA

CHAPTER 16: THE SPECTRUM OF ANTIMICROBIAL ACTIVITY IN BOVINE SEMINAL PLASMA.

The observation that bovine seminal plasma is effective against several bacterial species is extended here to cover the response of bacteria, mycoplasmas and ureaplasmas to bovine and human seminal plasma, lysozyme, polylysine,salmine and spermidine. These known antimicrobial agents were tested in conjunction with seminal plasma to determine whether the spectrum of antimicrobial activity and the response of a range of micro-organisms to these secretions resembled the spectrum of these selected compounds.

16.1 The Bacterial Response to Bovine and Human Seminal Plasma and to Selected Antimicrobial Agents.

The response of 90 bacterial strains (TABLE II) to bovine seminal plasma, human seminal plasma, lysozyme, polylysine, salmine and spermidine was tested as described in CHAPTER 8.2 , and the results recorded in APPENDIX FIVE . These results are summarised in TABLE XVI . A large proportion of the bacteria tested were resistant to human seminal plasma (43%), lysozyme (59%), and spermidine (60%) ; very few were resistant to salmine (2%) and polylysine (6%) . Bovine seminal plasma (18% resistant) was intermediate between these two groups.

The response of each bacterium tested against bovine seminal plasma and the selected inhibitors was recorded as showing sensitivity or resistance. The difference between bovine seminal plasma and a given inhibitor was expressed as the percentage of dissimilar responses in the total number of comparisons made (TABLE XVII). Bovine seminal plasma differed from human seminal plasma, lysozyme, polylysine, salmine and spermidine by 34, 50, 19, 20 and 49% respectively. Thus the inhibitory activity of bovine seminal plasma is unlikely to be identical with that of the other materials tested.

If the variation in an organism's response to bovine seminal plasma and to the other antimicrobial agents was based solely on the amount of material present, the diameter of the inhibition zone produced by bovine seminal plasma would be a constant proportion of that produced by the agent responsible for the inhibition. For example, bovine seminal plasma

TABLE XVI.

BACTERIAL RESISTANCE TO BOVINE SEMINAL PLASMA, HUMAN SEMINAL PLASMA, AND SELECTED ANTIMICROBIAL AGENTS.

GRAM-POSITIVE GENERA (number of strains tested)	NUMBER OF STRAINS RESISTANT TO : bovine seminal plasma	human seminal plasma	lysozyme	poly-lysine	salmine	spermidine
Arthrobacter (1)	0	0	0	0	0	0
Bacillus (8)	0	5	3	0	0	5
Corynebacterium (6)	0	5	4	0	0	2
Lactobacillus (6)	2	1	3	0	0	4
Leuconostoc (2)	0	0	1	0	0	1
Micrococcus (2)	0	0	0	0	0	0
Mycobacterium (1)	0	0	0	0	0	0
Nocardia (1)	1	1	1	1	0	1
Sarcina (1)	0	0	0	0	0	0
Staphylococcus (11)	1	3	10	0	0	3
Streptococcus (12)	5	5	8	2	2	7
Streptomyces (1)	0	0	0	0	0	0
TOTAL (52)	9	21	30	3	2	23
	17%	40%	58%	6%	4%	44%
GRAM-NEGATIVE GENERA						
Acetobacter (1)	0	0	1	0	0	1
Citrobacter (1)	0	1	1	0	0	1
Enterobacter (1)	0	1	1	1	0	1
Escherichia (9)	0	0	4	0	0	9
Haemophilus (1)	0	0	0	0	0	0
Klebsiella (3)	0	3	3	1	0	3
Neisseria (3)	0	0	0	0	0	0
Pasteurella (1)	0	0	1	0	0	0
Proteus (5)	5	5	3	2	0	5
Providence (1)	1	1	1	0	0	1
Pseudomonas (2)	1	2	1	0	0	2
Salmonella (6)	0	4	6	0	0	6
Serratia (1)	0	1	1	0	0	1
Shigella (2)	0	0	0	0	0	1
Vibrio (1)	0	0	0	0	0	0
TOTAL (38)	7	18	23	2	0	31
	18%	47%	61%	5%	0%	82%
TOTAL NUMBER OF BACTERIAL STRAINS (90)	16	39	53	5	2	54
	18%	43%	59%	6%	2%	60%

TABLE XVII.

A COMPARISON OF THE ANTIBACTERIAL ACTIVITY SPECTRA OF BOVINE SEMINAL PLASMA HUMAN SEMINAL PLASMA, AND SELECTED ANTIMICROBIAL AGENTS.

INHIBITOR	DISSIMILAR RESPONSES (%)	"D" VALUES* : Gram-Positive Bacteria	"D" VALUES* : Gram-Negative Bacteria
Bovine seminal plasma VERSUS			
Human seminal plasma	34	1.86	2.09#
Lysozyme	50	2.19#	3.74#
Polylysine	19	4.66#	1.61
Salmine	20	3.70#	2.03#
Spermidine	49	0.05	2.96#

FOOTNOTES : * D = $$\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

where there are n_1 responses to bovine seminal plasma having a mean $\bar{x}_1$ and an estimated variance s_1^2, and n_2 responses to a known inhibitor with mean $\bar{x}_2$ and variance s_2^2.

P is less than 0.05 when D is greater than 1.96. Therefore these results indicate significant differences between the two populations being studied (within 5% confidence limits).

produced a substantially bigger inhibition zone than lysozyme against Pseudomonas fluorescens. If this greater activity was simply due to larger amounts of lysozyme occurring in bovine seminal plasma than in the commercial preparation, other organisms sensitive to lysozyme would also show a greater response to bovine seminal plasma ; Proteus vulgaris , however, showed an inverse relationship, exhibiting a greater sensitivity to lysozyme than to bovine seminal plasma. Thus, the variation in the response of bacteria to bovine seminal plasma and lysozyme is not readily accounted for by differences in lysozyme concentration, and the antibacterial activity of bovine seminal plasma cannot be attributed solely to the presence of lysozyme. Similar situations exist between bovine seminal plasma and the other antimicrobial factors tested (APPENDIX FIVE). It is therefore proposed that differences in the sensitivity patterns of the various bacteria cannot be explained by concentration effects alone. Bovine seminal plasma showed no constant relationship with any of the antimicrobial agents tested and therefore, none of these agents is likely to be responsible for the antibacterial activity of bovine seminal plasma.

Since variations in bacterial response to bovine seminal plasma and the selected antimicrobial agents are not associated with simple concentration differences, the significance of differences in the means and standard deviations of the inhibition zone diameters produced by bovine seminal plasma and these agents can be tested. A "D" test (Bailey 1968) was used to determine whether bovine seminal plasma induced a bacterial response with a mean inhibition zone diameter sufficiently different from that induced by each test compound to indicate lack of identity. "D" is defined as

$$\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

where there are n_1 responses to bovine seminal plasma having a mean $\bar{x}_1$ and an estimated variance s_1^2 , and n_2 responses to a known inhibitor with mean $\bar{x}_2$ and variance s_2^2 . A calculated "D" value of 1.96 indicates that there was a difference between the two groups of responses at the 5% level of significance.

TABLE XVII shows that bovine seminal plasma differed significantly from lysozyme, polylysine and salmine in its effect on Gram-positive bacteria, but did not differ significantly from spermidine or human seminal plasma. For Gram-negative bacteria, inhibition by bovine seminal

plasma differed significantly from that of lysozyme, salmine, spermidine and human seminal plasma, but not from that of polylysine. Thus the diameter of the inhibition zone produced by bovine seminal plasma differed significantly from those produced by each of the selected antimicrobial agents tested with Gram-positive and/or Gram-negative bacteria.

Several genera of bacteria commonly associated with urogenital tract infections in cattle were found to include strains resistant to the bovine seminal plasma antibacterial activity. These included Proteus spp. (5 out of 5 strains tested were not inhibited by bovine seminal plasma), Providence sp. (1/1), Pseudomonas sp. (1/2) and Streptococcus spp. (5/12). Nocardia, some lactobacilli and staphylococci were also resistant to bovine seminal plasma. Among the 16 bacterial strains resistant to bovine seminal plasma (TABLE XVI), resistance to human seminal plasma, lysozyme and spermidine were exhibited by 13, 12 and 12 strains respectively. Only 3 strains showed growth in the presence of polylysine,and none were resistant to salmine.

16.2 The Effect of Bovine Seminal Plasma on Mycoplasmas and Viruses.

Bovine seminal plasma produced substantial inhibition zones (CHAPTER 8.3) with several Mycoplasma species : M. arginini, M. arthritidis, M. hominis (2 strains) and M. ovipneumoniae (2 strains). These mycoplasmas were not sensitive to lysozyme, polylysine, salmine or spermidine (TABLE XVIII). The activity of bovine seminal plasma against mycoplasmas was heat stable when heated to 60'C for 60 minutes (TABLE XII).

Two strains of T Mycoplasmas (Ureaplasmas) were inhibited by the incorporation of bovine seminal plasma into their U_9 growth medium (CHAPTER 8.3). Appropriate controls grew readily in the absence of bovine seminal plasma. No ureaplasmas could be recovered on subculture from the test broths, indicating the presence of a mycoplasmicidal activity in bovine seminal plasma.

Subsequently bovine seminal plasma was found to inactivate several enveloped viruses, including Herpes viruses 1 and 2 , and the Infectious Bovine Rhinotracheitis (IBR) virus (personal communication, Clarke 1976).

TABLE XVIII.

MYCOPLASMAL SENSITIVITY TO BOVINE SEMINAL PLASMA, HUMAN SEMINAL PLASMA AND SELECTED ANTIMICROBIAL AGENTS.

MYCOPLASMA	DIAMETER OF INHIBITION ZONE (MM) WITH :					
	bovine seminal plasma	human seminal plasma	lysozyme	poly-lysine	salmine	spermi-dine
M. arginini	23.00 (47,500 LzU_e)	0	0	0	0	0
M. arthritidis	25.50 (91,000 LzU_e)	0	0	0	0	0
M. hominis A	27.00 (132,500 LzU_e)	0	0	0	0	0
M. hominis B	26.00 (102,500 LzU_e)	0	0	0	0	0
M. ovipneumoniae A	25.00 (81,000 LzU_e)	0	0	0	0	0
M. ovipneumoniae B	23.50 (55,000 LzU_e)	0	0	0	0	0

CHAPTER 17: THE MODE OF ACTION OF THE ANTIMICROBIAL ACTIVITY OF BOVINE SEMINAL PLASMA.

17.1 The Microscopic Examination of Bacteria Treated with Bovine Seminal Plasma.

A suspension of Micrococcus lysodeikticus cells which gave a reading of 20 units in a Klett - Summerson colorimeter fitted with a blue filter (400 to 460nm) was prepared from a 24 hour culture in nutrient broth and examined as a wet preparation under the light microscope (x 400). To test the effect of semen proteins on bacteria, bovine seminal plasma or a crude seminal plasma extract (5% w/v solution of the acetone precipitated acid dialysate, CHAPTER 18.1), was allowed to perfuse from one side of the coverslip to the other. Cellular morphology was examined under these conditions for 1 hour : no lysis occurred and cellular definition remained good. When a 5% w/v solution of egg white lysozyme was used for comparison, the bacterial cells aggregated and distinction between individual cells in these aggregates became less apparent. The addition of the crude extract of bovine seminal plasma proteins to the cell suspension resulted in the appearance of some amorphous material ; this could have been interpreted as lysed cell debris, except that this material was also produced in the absence of bacterial cells when the extract was added to pure nutrient broth. This may represent medium constituents precipitated from the broth solution. Intact cells were always present within this debris when bovine seminal plasma was added to micrococcal cell suspensions.

A study of the sensitivity of bacteria at different stages of growth failed to reveal any alteration in the response of M. lysodeikticus to bovine seminal plasma proteins. No lysis occurred within 1 hour when crystals of bovine seminal plasma extract (CHAPTER 18.1) were added to 4 samples of cells in the logarithmic growth phase and 4 samples of cells from early to late stationary growth phase.

Leptospira pomona and L. hardjo were grown in E.M.J.H. medium (Bacto Leptospira Enrichment EMJH, Difco) for 10 days at 25'C, treated with bovine seminal plasma proteins and examined under dark field illumination for morphological changes caused by contact with seminal plasma. While a slide of a leptospira culture was under the microscope, a 5% w/v solution of acetone precipitated acid dialysate of bovine seminal plasma (CHAPTER 18.1) was infused into the culture fluid from one side of a coverslip. In a second experiment, crystals of the same extract were placed in a drop of leptospira culture,the drop was covered with a coverslip and the organism was examined for visible alterations. The crystals

were seen to dissolve in the culture fluid, but the organisms remained active for at least two hours in regions where the crystals had dissolved. There was a slight increase in the apparent width of the bacteria but little else was observed ; the leptospira appeared to be resistant to the bovine seminal plasma antimicrobial effects.

17.2 Bacteriostatic versus Bacteriocidal Effects of Bovine Seminal Plasma.

The effect of bovine seminal plasma on bacterial cells was examined by the method described in CHAPTER 8.4 , to determine the number of bacteria surviving after increasing periods of time in contact with the antimicrobial material isolated from bovine seminal plasma by adsorption onto DNA (CHAPTER 19.4).

Initially this assay was performed with Micrococcus lysodeikticus as the test organism ; brain heart infusion (BHI) broth was used as a diluent and BHI agar as the growth medium to determine viable counts. The response of M. lysodeikticus to 10mg/cm^3 of DNA extracted material has been plotted in FIGURE TWENTY SIX. The initial slower rate of death might be due to a protective effect associated with BHI broth components, or to the occurrence of pairs or groups of micrococcal cells which have generated a two -hit kill curve. To eliminate the former possibility, further studies were undertaken using a less rich medium, i.e. nutrient broth. In all these subsequent experiments there was no trace of a similar lag period before bacteriocidal activity occurred. Death occurred rapidly in each population examined. Thus BHI medium may act as a non specific inhibitor of the antibacterial activity of the DNA extract, decreasing its effectiveness against sensitive bacterial cells by adsorption and inactivation of the active principle, or by enabling some repair of the initial damage to the cells.

Three other bacteria sensitive to bovine seminal plasma by the agar well diffusion assay were tested in addition to M. lysodeikticus using nutrient broth medium (FIGURE TWENTY SEVEN). M. lysodeikticus in nutrient broth was rapidly killed by bovine seminal plasma DNA extract (5mg/cm^3) with no slow initial rate of kill. Bovine seminal plasma DNA extract (5mg/cm^3) also significantly decreased the viable population of Escherichia coli and Pseudomonas fluorescens. Its most rapid kill was of E. coli cells. Further studies are necessary to explain the apparent paradox in which M. lyosdeikticus, a more sensitive bacterium according to the agar well diffusion assay (20.5mm inhibition zone diameter,APPENDIX FIVE) was less rapidly killed by bovine seminal plasma DNA extract in

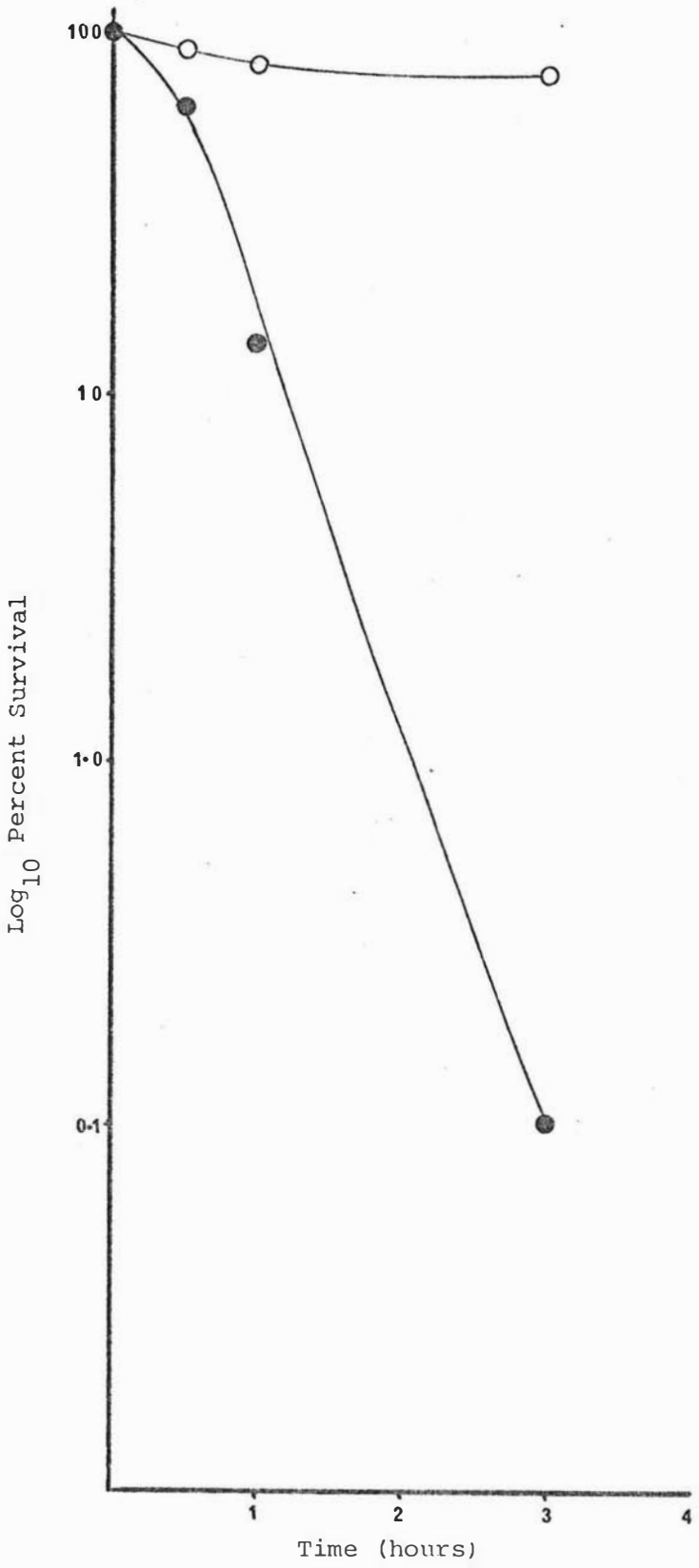


FIGURE TWENTY SIX: Percentage Survival in BHI broth of Micrococcus lysodeikticus in the Presence of 10mg/cm^3 (●) DNA-isolated Material From Bovine Seminal Plasma (CHAPTER 19.4) compared with Control Growth (○).

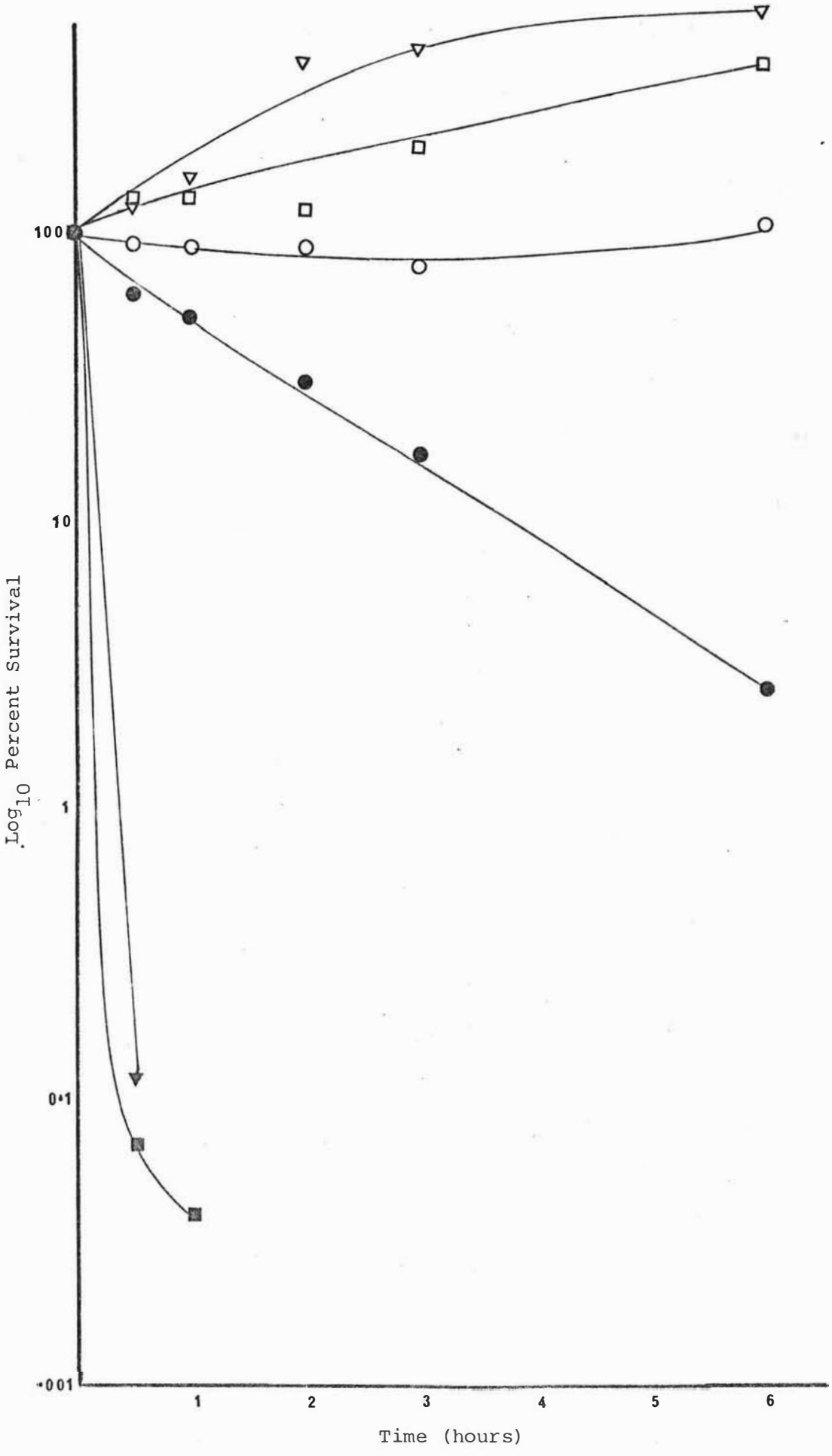


FIGURE TWENTY SEVEN:

nutrient broth studies than E. coli (16.75mm inhibition zone diameter, APPENDIX FIVE) and Ps. fluorescens (17.25mm inhibition zone diameter, APPENDIX FIVE).

The bovine seminal plasma DNA extract (5mg/cm^3) had no bactericidal activity against Bacillus subtilis in nutrient broth. Initially the bacterial growth in the test culture was retarded compared with the control culture in the absence of DNA extract, but the variation was slight,and within 3 hours no difference existed between test and control populations (FIGURE TWENTY EIGHT). This supports the evidence in CHAPTER 13.3, TABLE XII, that the activity of bovine seminal plasma against B. subtilis is associated with a different heat labile compound, rather than the heat stable component responsible for the activity against M. lysodeikticus, E. coli etc.

17.3 A Comparison of the Antibacterial Activity of Bovine Seminal Plasma and of the Activity of a Selection of Commercially Available Antibiotics.

Bacterial resistance to antibiotics is often associated with an alteration of a cellular component so that the antibiotic either does not reach or does not react in a typical manner at its target site within the bacterial cell. In such a case, a mutation resulting in resistance to an antibiotic active at a specific target site may also enhance the cell's resistance to antibiotics functioning in a similar manner or affecting the same target site. For example, phenethyl alcohol is considered to attack the cell membrane with a consequent effect on DNA synthesis. A single gene is responsible for conferring resistance to acriflavine and phenethyl alcohol in Escherichia coli (Scott Foster & Russell 1971) and it has been postulated that resistance to acriflavine is at the membrane level also. Similar correlations have been used in this study to obtain some indication of the nature of the antimicrobial activity in bovine seminal plasma on bacterial cells.

The response patterns of 53 strains of bacteria to bovine seminal plasma and to a selected group of commercial antibiotics are recorded in APPENDIX FOUR. The antibiotics have been divided into four groups on the basis of their mode of action. Bacitracin, cycloserine, penicillin and ristocetin are all cell wall associated antibiotics. Colistin and polymyxin affect membrane integrity and sulfafurazole is a metabolic analogue. The other five antibiotics (chloramphenicol, erythromycin, kanamycin, streptomycin and tetracycline) all interfere with protein synthesis. The bacterial responses to these four antibiotic groups are summarised in TABLE XIX. For a comparison of the responses of these organisms to bovine

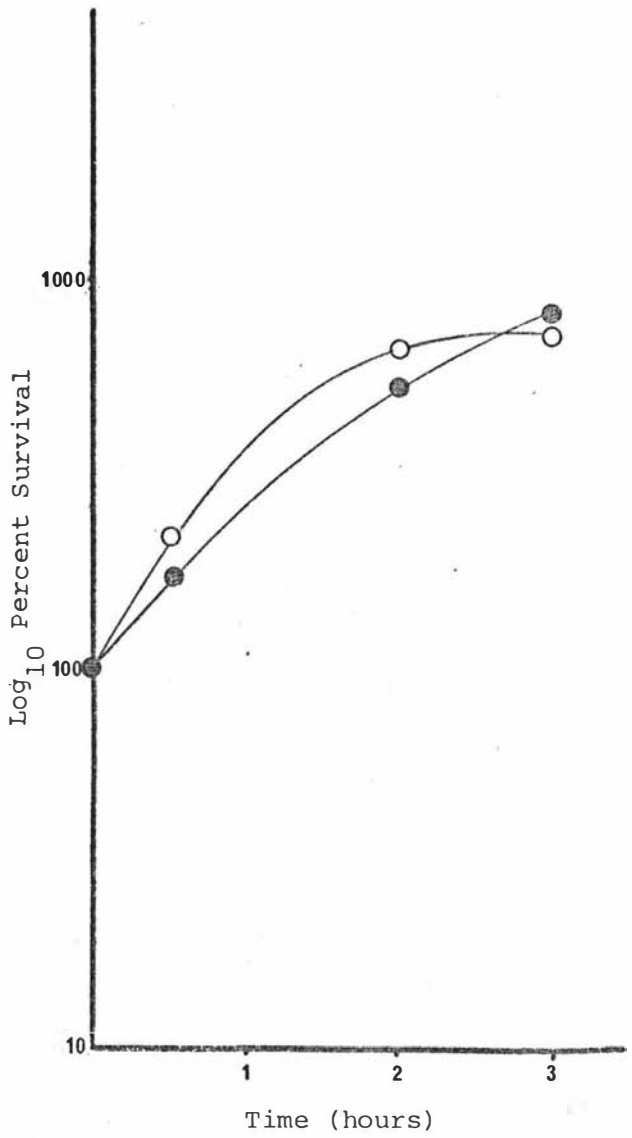


FIGURE TWENTY EIGHT: The Effect of 5mg/cm^3 DNA-isolated Material from Bovine Seminal Plasma on Bacillus subtilis in Nutrient Broth (●) , compared with Control Growth in absence of DNA-isolated Material (○).

TABLE XIX.

THE CORRELATION BETWEEN RESISTANCE TO BOVINE SEMINAL PLASMA AND RESISTANCE TO A VARIETY OF ANTIBIOTICS.

BACTERIUM	NUMBER OF STRAINS	NUMBER OF RESISTANT RESPONSES TO : bovine seminal plasma	cell wall antibiotics (4)	membrane antibiotics (2)	protein synthesis antibiotics (5)	metabolic analogue (1)
		%	%	%	%	%
Bacillus spp.						
sensitive	7	0/ 7 (0)	7/28 (25)	0/14 (0)	0/35 (0)	2/ 7 (29)
resistant	2	2/ 2 (100)	2/ 8 (25)	2/ 4 (50)	1/10 (10)	2/ 2 (100)
Corynebacterium spp.						
sensitive	4	0/ 4 (0)	8/16 (50)	1/ 8 (13)	2/20 (10)	3/ 4 (75)
resistant	3	3/ 3 (100)	6/12 (50)	4/ 6 (67)	2/15 (13)	1/ 3 (33)
Gram-positive Bacteria						
sensitive	21	0/21 (0)	24/84 (29)	6/42 (14)	5/105(5)	12/21 (57)
resistant	6	6/ 6 (100)	8/24 (33)	7/12 (58)	3/30 (10)	4/ 6 (67)
Gram-negative Bacteria						
sensitive	4	0/ 4 (0)	14/16 (88)	0/ 8 (0)	6/20 (30)	4/ 4 (100)
resistant	6	6/ 6 (100)	24/24 (100)	2/12 (17)	15/30 (50)	6/ 6 (100)

seminal plasma and to the various antibiotics, the variations in the number of resistant organisms to each antibiotic group were examined with reference to their resistance to bovine seminal plasma.

Among Bacillus species, bovine seminal plasma resistant strains exhibited a higher frequency of resistant responses to antibiotics affecting bacterial cell membranes and to the metabolic analogue, sulfafurazole, than did the strains sensitive to bovine seminal plasma. There was no significant increase in the number of resistant responses to antibiotics affecting protein synthesis and cell wall synthesis between bacteria sensitive to and those resistant to bovine seminal plasma.

Corynebacterium species which were resistant to bovine seminal plasma, also showed an increased frequency of resistance to cell membrane associated antibiotics. There was no significant increase in the frequency of resistance to antibiotics affecting cell wall synthesis, protein synthesis or folic acid metabolism.

For those bacterial genera which did not show a variation in their response to bovine seminal plasma, less definitive conclusions could be reached. It was clear, however, that Gram-positive bacteria resistant to bovine seminal plasma showed a significant concomitant increase in the frequency of resistance to antibiotics affecting cell membrane integrity. Among Gram-negative bacteria, those resistant to bovine seminal plasma also exhibited an increased frequency of resistance to antibiotics affecting cell wall synthesis, cell membrane integrity and protein synthesis.

In summary, it is apparent that resistance to bovine seminal plasma failed to show a consistent relationship with responses to antibiotics associated with cell wall development, protein synthesis, or to the metabolic analogue, sulfafurazole. A significant relationship did, however, occur between resistance to bovine seminal plasma and an increased frequency of resistance to antibiotics affecting cell membrane integrity. This supports the view that the cell membrane may be the focus of attack by the antimicrobial factor present in bovine seminal plasma.

17.4 Further Evidence for the Cell Membrane being the Site of Action for the Antimicrobial Activity in Bovine Seminal Plasma.

This work was undertaken by Dr B.D.W. Jarvis, Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand.

In the presence of bovine seminal plasma, bacteria sensitive to its antimicrobial activity (Bacillus megaterium and Micrococcus lysodeikticus) rapidly lost accumulated ^{14}C amino-iso-butyric acid, a non

metabolisable analogue of glycine and alanine.

In addition, bovine seminal plasma completely prevented the accumulation of ^{14}C valine, when the seminal plasma and ^{14}C valine were added simultaneously to a suspension of B. megaterium cells. Seminal plasma also slowed the rate of accumulation of ^{14}C valine by a suspension of Pseudomonas aeruginosa cells, although these cells were resistant to bovine seminal plasma in the radial diffusion assay (CHAPTER 4.3).

In Gram-negative bacteria, there was a relatively nonspecific enhancement of resistance to a variety of antibiotics which was also associated with resistance to bovine seminal plasma. This was thought to be due to a general decrease in the permeability of the exterior lipo polysaccharide cell layer in these cells. This hypothesis was supported by an examination of the effect of bovine seminal plasma on cellular contents. On addition of bovine seminal plasma, there was a massive leakage of cytoplasmic material from suspensions of sensitive cells. This leakage was readily seen as an increase in the absorbance of the supernatant at 240 to 290nm. This increase was not attributable to the absorbance of bovine seminal plasma, and differed from the material released by the French press which had greater absorbance between 250 to 270nm. Ps. aeruginosa, a resistant organism, showed less leakage than the sensitive bacteria in the presence of bovine seminal plasma, but some degree of damage to cell membrane integrity was apparent even in these cells.

When osmotically sensitive cells were prepared by lysozyme treatment of B. megaterium and M. lysodeikticus, exposure of these cells to bovine seminal plasma caused immediate and complete lysis. No intact cells could be observed. Osmotically sensitive cells of the resistant Ps. aeruginosa also underwent immediate and complete lysis on addition of bovine seminal plasma. Thus, damage to cell wall integrity can render a formerly resistant bacterium sensitive to the action of bovine seminal plasma.

There was no evidence of damage to the cell walls of B. megaterium and M. lysodeikticus after contact for 30 minutes with bovine seminal plasma. Microscopic examination showed intact cells staining rather more lightly with methylene blue after exposure to the bovine seminal plasma.

RESULTS : PART FOUR

THE ISOLATION AND CHARACTERISATION OF AN ANTIMICROBIAL FACTOR FROM BOVINE SEMINAL PLASMA.

CHAPTER 18: PRELIMINARY ATTEMPTS TO ISOLATE A FACTOR RESPONSIBLE FOR THE ANTIMICROBIAL ACTIVITY OF BOVINE SEMINAL PLASMA.

18.1 Acid Dialysis and Acetone Precipitation to give a Crude Preparation of an Antimicrobial Compound present in Bovine Seminal Plasma.

Dialysis of bovine seminal plasma adjusted to pH 2.5 with HCl against an equal volume of distilled water adjusted to pH 2.5 with HCl for 3 days at 4'C, resulted in 14% of the activity diffusing through the membrane (CHAPTER 15.2). The active material in the dialysate was precipitated by the addition of 8 volumes of acetone. This precipitate was collected by centrifugation at 4'C and 12,000 rpm for 20 minutes. No activity remained in the supernatant. The precipitate was dried in a vacuum desiccator over concentrated H_2SO_4 at 4'C for 4 days.

The protein content of whole seminal plasma and the precipitated material were determined by Kjeldahl-Gunning digestion and the Crismer modified Van Slyke & Hiller procedure for ammonium determination (CHAPTER 9.1). The specific activity of the precipitated material showed a three-fold increase over that of whole seminal plasma (TABLE XX).

The absorbance spectrum of this preparation was similar to the spectrum obtained with whole bovine seminal plasma (FIGURE TWENTY TWO , CHAPTER 15.1).

Native polyacrylamide gel electrophoresis (CHAPTER 7.5) gave multiple bands of both cationic and anionic material at pH 8.6 . Two major cationic bands were observed and recorded by densitometric tracings of the slab gel ; these bands were distinct from those exhibited by whole bovine seminal plasma and lysozyme (CHAPTER 15.4 , FIGURE TWENTY THREE). By overlaying the gel with nutrient agar seeded with M. lysodeikticus (CHAPTER 7.6) it could be demonstrated that the antibacterial activity of this preparation was associated with at least two of the cationic protein bands (CHAPTER 15.4 , FIGURE TWENTY FOUR (a) and (b) and FIGURE TWENTY FIVE (a) and (b)). Several other protein bands were also present but these were not observed to exhibit antibacterial activity.

The above acid dialysis and acetone precipitation was used to obtain partially purified material which could be employed to investigate other

TABLE XX.

THE ANTIBACTERIAL ACTIVITY, NITROGEN CONTENT AND SPECIFIC ACTIVITY OF ACETONE PRECIPITATED ACID DIALYSATE MATERIAL AND WHOLE BOVINE SEMINAL PLASMA

MATERIAL	ANTIBACTERIAL ACTIVITY*	NITROGEN CONTENT (mg N / cm^3)	SPECIFIC ACTIVITY (LzU_e / mg N)
bovine seminal plasma	3.6×10^4	9.10	3.96×10^3
precipitate	6.4×10^3	0.57	1.12×10^4

FOOTNOTE: * Activity expressed as lysozyme unit equivalents (LzU_e), see CHAPTER 4.4 .

techniques for purifying the compound(s) responsible for the antimicrobial activity of bovine seminal plasma.

18.2 Ion Exchange Chromatography of Bovine Seminal Plasma.

Native polyacrylamide gel electrophoresis of bovine seminal plasma and the acetone precipitated material from acid dialysis of bovine seminal plasma (CHAPTER 15.4) showed that the antimicrobial activity was associated with proteins which migrated as cationic moieties at pH 8.6 . It was therefore decided to investigate the use of cationic exchange resins to separate the active compounds from other proteins in bovine seminal plasma.

18.2a Choice of Cationic Resin and Optimum Conditions for the Adsorption of Antimicrobial Activity from Bovine Seminal Plasma.

Five cationic exchange resins were tested at five different pHs, to determine which resin adsorbed the greatest amount of antimicrobial activity and the least amount of extraneous protein from the partially purified (acetone precipitated acid dialysate of bovine seminal plasma, CHAPTER 18.1) preparation of the antimicrobial factor(s) in bovine seminal plasma. The resins tested,the buffers chosen and the method used in this study are described in CHAPTER 9.2a , CHAPTER 9.2b and CHAPTER 9.2c .

The absorbance of supernatants from solutions of this material after contact with the various resins to be tested was recorded (CHAPTER 9.2), and the percent protein adsorbed by each resin at each pH was calculated using the absorbance at 277nm as a measure of the remaining protein,which was not adsorbed by the resin. These values are recorded in TABLE XXI . Values for absorbance at pH 8.6 were not possible as the barbiturate buffer used at this pH interfered with the absorbance at 277nm.

Each supernatant was run by native polyacrylamide gel electrophoresis (CHAPTER 7.5) following contact with a given resin to determine whether specific protein bands had been removed by that resin (FIGURE TWENTY NINE). Dowex 50 - XA adsorbed little of the proteins present, only noticeably decreasing those proteins most highly cationic. Amberlite IR-120 and Whatman CM_{32} removed the major protein peak as well as some of the most rapidly migrating protein, but the slower moving peaks remained intact. Amberlite IRC-50 and Sephadex SP_C25 removed large amounts of most of the protein present, although the faster moving peaks were more apparent in the Sephadex treated supernatant than in the Amberlite IRC-50 treated sample.

The supernatants were also tested for antibacterial activity using the radial diffusion assay with M. lysodeikticus as the indicator organism (CHAPTER 4.3). The percent activity adsorbed by each resin was calculated

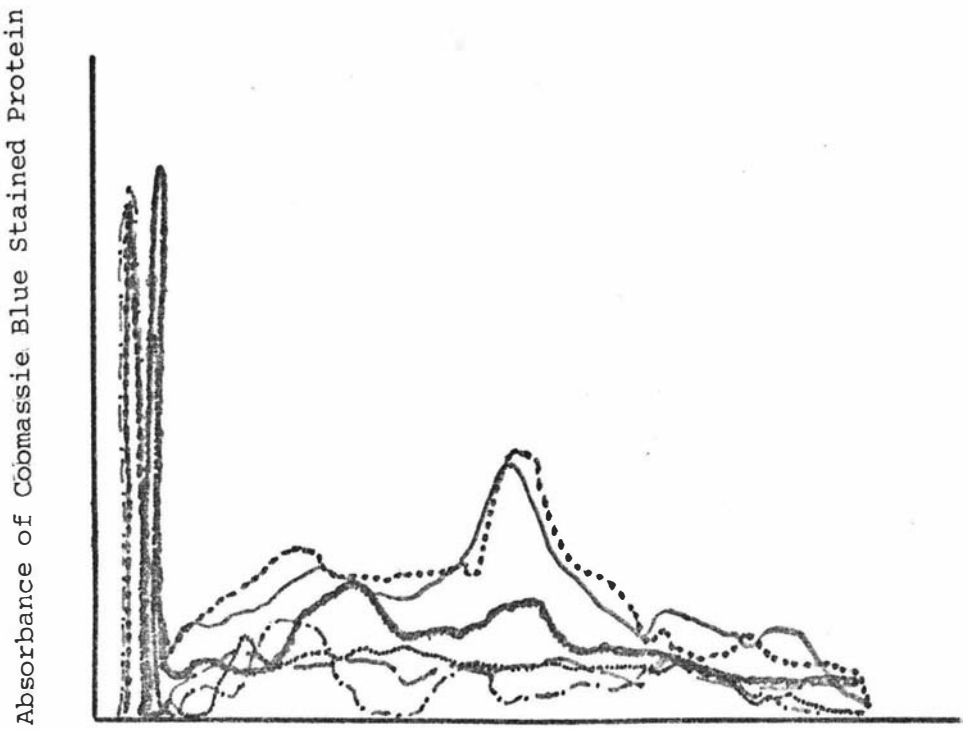


FIGURE TWENTY NINE: Densitometer Tracings of pH 8.6 Native Polyacrylamide Gel Electrophoresis Slabs for Cationic Proteins in Partially Purified (acetone precipitated acid dialysate CHAPTER 18.1) Antimicrobial Material from Bovine Seminal Plasma after Contact with Cation Exchange Resins.

= Untreated Antimicrobial material
= Supernatant after contact with Sephadex SP_C 25
= " " " " Dowex 50-XA
= " " " " Amberlite IRC-50
= " " " " Amberlite IR-120
= " " " " Whatman CM32 .

from the amount of activity present in the original sample and the amount remaining in the supernatant after contact with the resin (TABLE XXI).

From these results it can be concluded that Amberlite IRC-50 and Whatman CM_{32} were both able to adsorb all of the antibacterial activity present in the partially purified preparation of bovine seminal plasma proteins in the 30 minute contact time allowed. This activity is located in the more rapidly moving proteins under conditions of native polyacrylamide gel electrophoresis, since it is these proteins which disappeared in all instances where activity had been adsorbed by the resin.

It was decided to use Amberlite IRC-50 resin for the cationic exchange chromatography of bovine seminal plasma proteins, since this resin required no pretreatment prior to equilibration with the appropriate buffer and was available in a bead form which possessed enhanced flow properties compared to the Whatman CM_{32} ion exchange resin. The optimum pH was chosen as that which allowed maximum adsorption of the antibacterial activity with minimum protein adsorption. Complete adsorption of the active fraction with lowest non-specific protein adsorption occurred at pH 8.0 .

18.2b Ion Exchange Chromatography of Bovine Seminal Plasma Proteins.

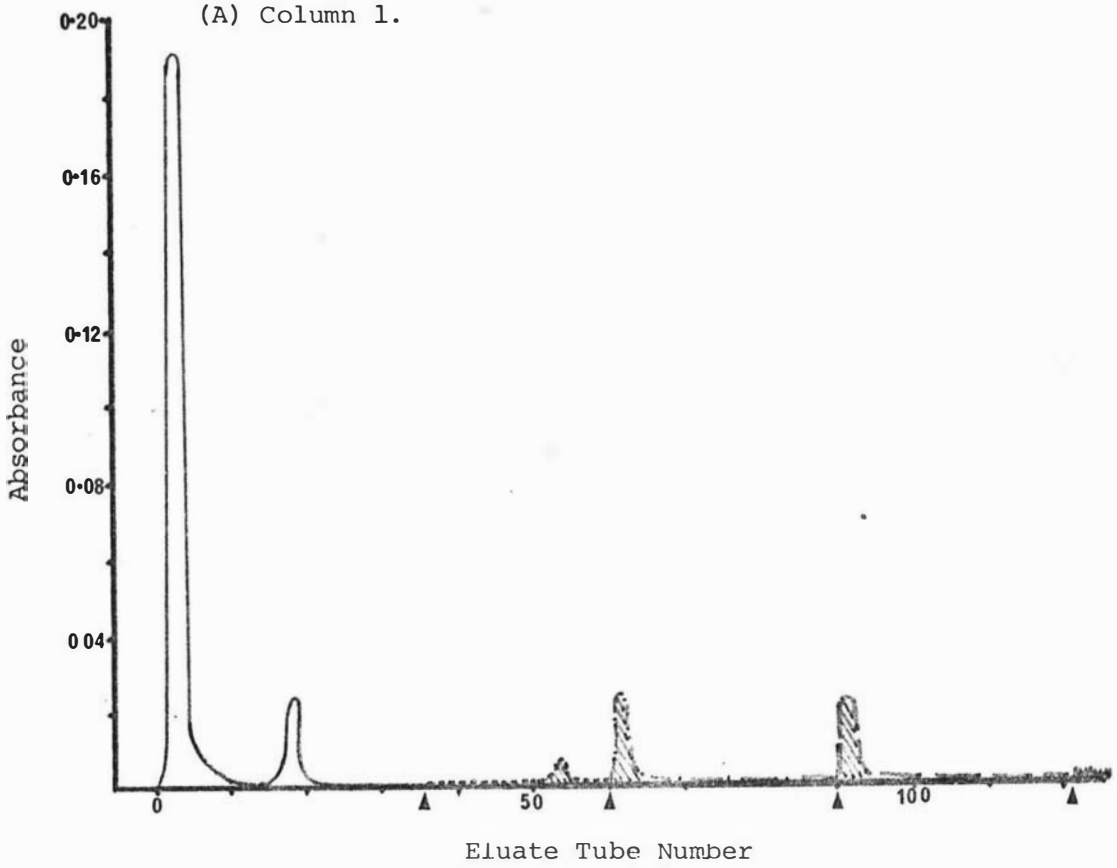
Two columns (CHAPTER 9.2d) were packed with Amberlite IRC-50 resin equilibrated with pH 8.0 Tris / HCl buffer, loaded with partially purified (CHAPTER 18.1) bovine seminal plasma proteins, and then the adsorbed proteins were eluted in a stepwise method off the ion exchange resin. The results of absorbance studies and antibacterial assays on aliquots taken from the collected fractions are recorded in FIGURE THIRTY. At least three protein peaks eluted off the cationic exchange resin at different ionic strengths were found to exhibit antibacterial activity.

This variation in the affinity of the antibacterial factor(s) may have been associated with the multivalent type of buffer used as an eluant. It was therefore decided to investigate the cationic exchange characteristics of a similar column using a simple anionic / cationic buffer (Ph 8.0 phosphate buffer). This eliminated any variation in the affinity of the antibacterial factor(s) associated with interactions between the compound responsible for the activity and the Tris molecules in the original buffer. Using 0.005M Na_2HPO_4 / NaH_2PO_4 buffer,followed by stepwise elutions with 0.01M, 0.05M, 0.1M, 0.2M and 0.4M solutions, similar results were obtained in the protein profiles and antibacterial activity locations of the eluates to those obtained using the Tris / HCl

TABLE XXI.

THE PERCENTAGE OF PROTEIN AND ANTIBACTERIAL ACTIVITY ABSORBED BY CATION EXCHANGE RESINS AT VARYING PH.

RESIN	PH	PERCENT PROTEIN ADSORBED (% Δ in OD_{277})	PERCENT ACTIVITY ADSORBED (% Δ in Activity)
Amberlite IR-120	7.6	0	22
	8.0	0	64
	8.6	-	50
	9.0	0	53
	9.6	8	37
Amberlite IRC-50	7.6	33	100
	8.0	26	100
	8.6	-	100
	9.0	26	100
	9.6	36	100
Dowex 50-XA	7.6	3	67
	8.0	8	64
	8.6	-	21
	9.0	6	52
	9.6	13	24
Sephadex SP_C25	7.6	36	- 42
	8.0	48	- 28
	8.6	-	- 57
	9.0	34	- 58
	9.6	39	- 42
Whatman CM_{32}	7.6	53	100
	8.0	34	100
	8.6	-	100
	9.0	24	100
	9.6	42	100



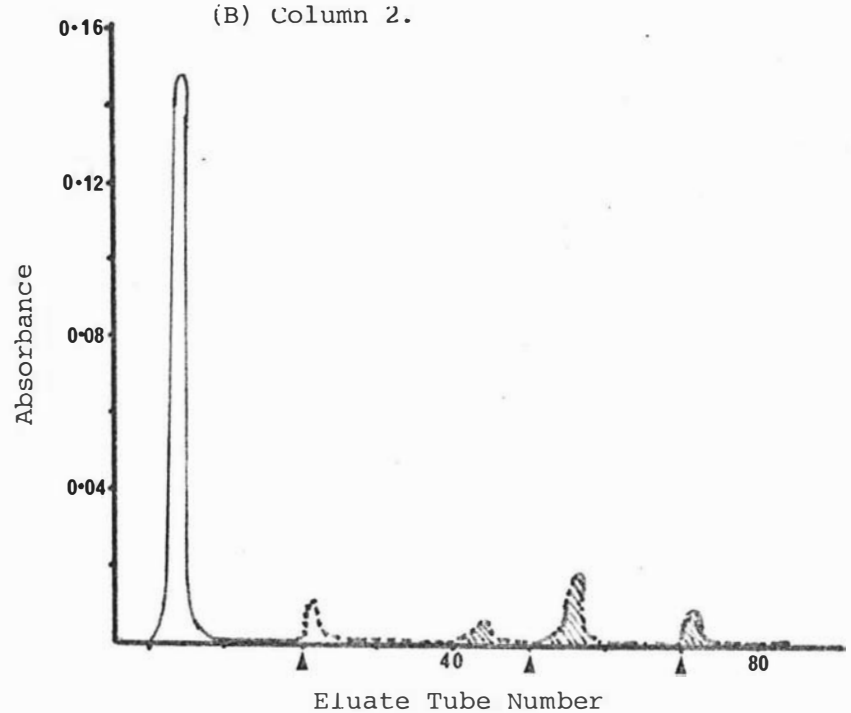


FIGURE THIRTY: Amberlite IRC-50 Ion Exchange Chromatography (CHAPTER 18.2) of Bovine Seminal Plasma Proteins with stepwise elution of pH 8.0 Tris/Hcl buffer of increasing ionic strength : ( )= 0.05M ; ( )= 0.075M ; ( )= 0.10M ; ( )= 0.20M ; and ( )= 0.50M elution buffer.

Shaded areas ( ) under protein peaks denote samples with Antibacterial Activity.

buffer system (FIGURE THIRTY ONE). A larger protein peak was eluted with 0.05M phosphate buffer than with similar strength Tris buffer ; the anti-bacterial activity was not confined to this peak ,but again occurred in three separate protein peaks from eluates of different ionic strengths.

18.2c The Effect of Sephadex SPc 25 Treatment on Bovine Seminal Plasma Proteins.

It is of interest to note that when Sephadex SP_C25 cation exchange resin was used to treat a partially purified preparation of bovine seminal plasma proteins (Acetone precipitated acid dialysate, CHAPTER 18.1),the activity of the treated material was greater than that of the original sample, although from protein absorption studies it was clear that the resin had adsorbed substantial amounts of protein from the solution (TABLE XXI). The maximum enhancement of antibacterial activity occurred at pH 9.0 ; the supernatant after adsorption with Sephadex SP_C25 had 158% of the activity and 66% of the protein as compared with the original untreated material. On native polyacrylamide gel electrophoresis (CHAPTER 7.5), the major cationic peak present in the untreated preparation was absent after Sephadex SP_C25 treatment, and although cationic material was present there was little banding, rather, there was a continuous migration from the source. (FIGURE TWENTY NINE).

A further study, using 1g of resin equilibrated to pH 9.0 in boric acid / borate buffer to treat $6cm^3$ of a $7mg/cm^3$ solution of partially purified material (CHAPTER 18.1), resulted in a 5x increase in the antibacterial activity of the treated material (TABLE XXII).

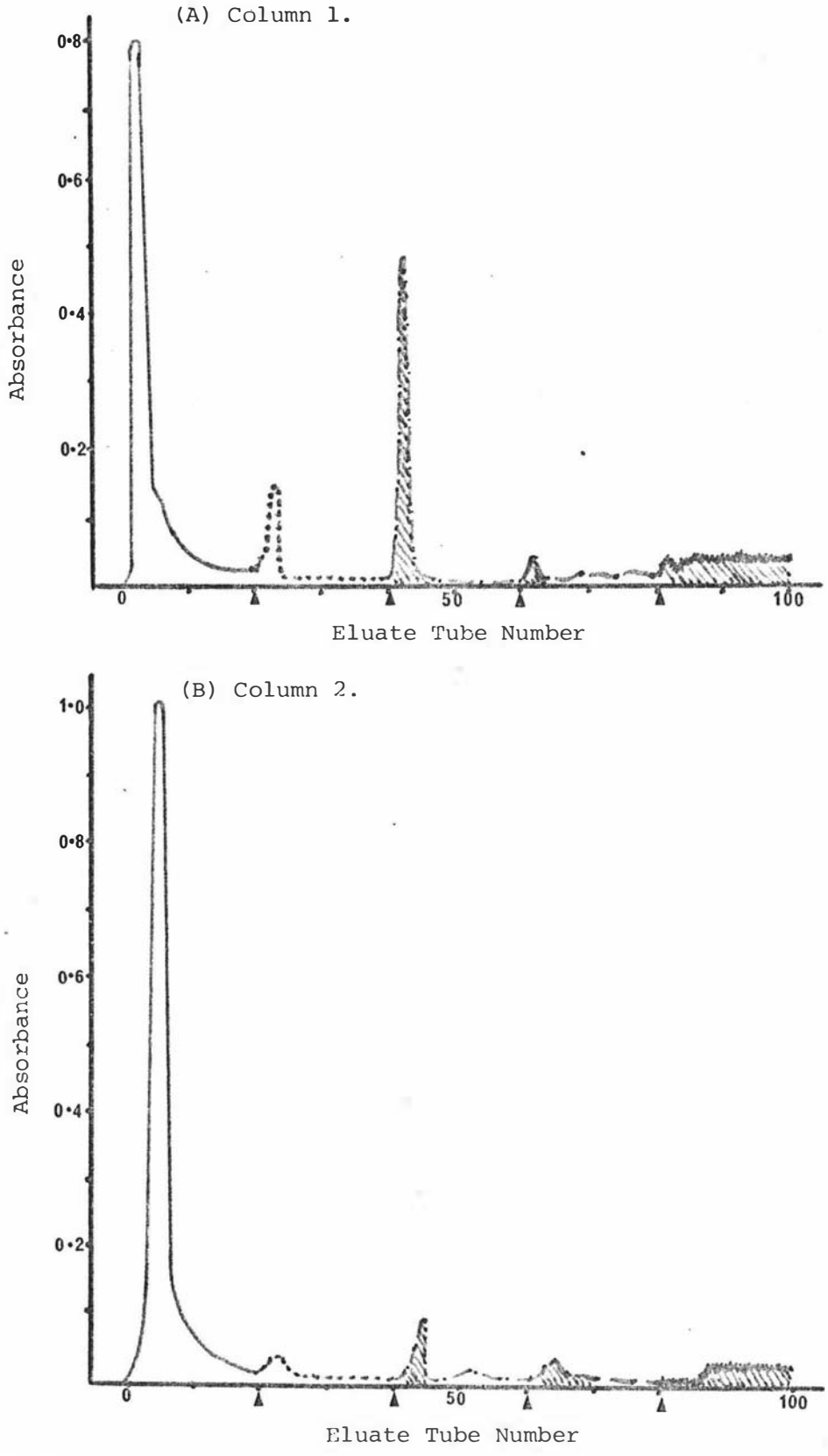


FIGURE THIRTY ONE: Amberlite IRC-50 Ion Exchange Chromatography (CHAPTER 18.2) of Bovine Seminal Plasma Proteins with stepwise elution of pH 8.0 phosphate buffer of increasing ionic strength : ( )= 0.005M ; ( )= 0.01M ; ( )=).05M ; ( )= 0.10M ; and ( )= 0.20M elution buffer.

Shaded areas ( ) under protein peaks denote samples with Antibacterial Activity.

TABLE XXII.

THE EFFECT OF SEPHADEX TREATMENT ON THE ANTIBACTERIAL ACTIVITY OF BOVINE SEMINAL PLASMA PROTEINS.

PH	ORIGINAL ACTIVITY*	ACTIVITY* IN SUPERNATANT AFTER SEPHADEX TREATMENT
7.6	9,000	12,750 (142%)
8.0	10,000	12,750 (128%)
8.6	10,000	14,300 (143%)
9.0	9,000	14,250 (158%)
9.6	7,900	11,250 (142%)
repeat		
9.0	27,750	140,000 (505%)

FOOTNOTE : * activity expressed as lysozyme unit equivalents (LzU_e), see CHAPTER 4.4 .

CHAPTER 19: DEOXYRIBONUCLEIC ACID (DNA) ISOLATION OF A COMPOUND RESPONSIBLE FOR SOME OF THE ANTIMICROBIAL ACTIVITY OF BOVINE SEMINAL PLASMA.

Once it was observed that the antibacterial activity in bovine seminal plasma exhibited varying affinities for the ion exchange resin, Amberlite IRC-50, attempts were made to find a material to which the antibacterial activity might have a strong and highly specific binding relationship. The compound(s) under investigation was of a cationic nature, and it was decided to explore its affinity for deoxyribonucleic acid (DNA); it was possible that the compound(s) involved might imitate the histones in their highly specific interaction with DNA, a multi-anionic biopolymer.

19.1 Conditions for the Adsorption of the Antibacterial Activity onto DNA.

The adsorption by DNA of antibacterial activity from bovine seminal plasma was tested under a variety of conditions to determine those which allowed adsorption of the greatest amount of antibacterial activity by a given amount of DNA (CHAPTER 10.1).

19.1a The Effect of pH on the Ability of DNA to Adsorb the Active Factor from Bovine Seminal Plasma.

Bovine seminal plasma at pH 6.0, 7.0 and 8.0 was treated with varying amounts of DNA and the amounts of antibacterial activity adsorbed in each treatment was calculated (CHAPTER 10.1), by measuring the decrease in activity in the supernatant following adsorption. As can be seen in FIGURE THIRTY TWO, DNA adsorbed the greatest amounts of antibacterial activity at pH 8.0 .

19.1b The Buffering Effect of DNA.

The addition of DNA to bovine seminal plasma resulted in a change in pH. The amount of 0.2M Na_2HPO_4 / NaH_2PO_4 pH 8.0 buffer required to hold a 5mg/cm^3 DNA solution at pH 8.0 was determined. The results included in FIGURE THIRTY THREE, established that 0.15cm^3 buffer per 1cm^3 DNA solution will maintain the pH following the addition of DNA to bovine seminal plasma. This amount of buffer was therefore added to each seminal plasma sample after adjustment to pH 8.0 with NaOH, to ensure a constant pH throughout the period of adsorption.

19.1c Amount of DNA Required for Maximum Adsorption of Antibacterial Activity at pH 8.0.

Increasing amounts of DNA resulted in increased amounts of activity being adsorbed from pH 8.0 buffered bovine seminal plasma at room

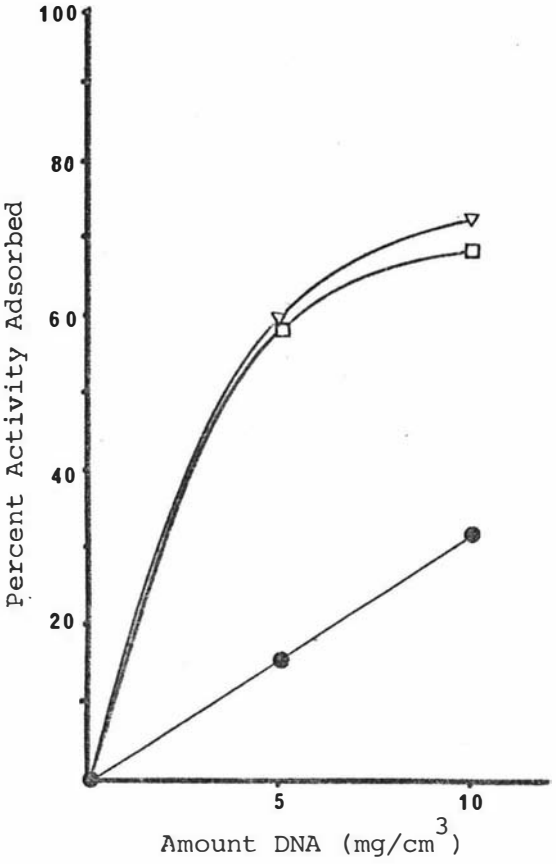


FIGURE THIRTY TWO: The Effect of pH (6.0 = ● , 7.0 = □ , 8.0 = ▽) on the Amount of Antibacterial Activity Adsorbed by DNA from Bovine Seminal Plasma.

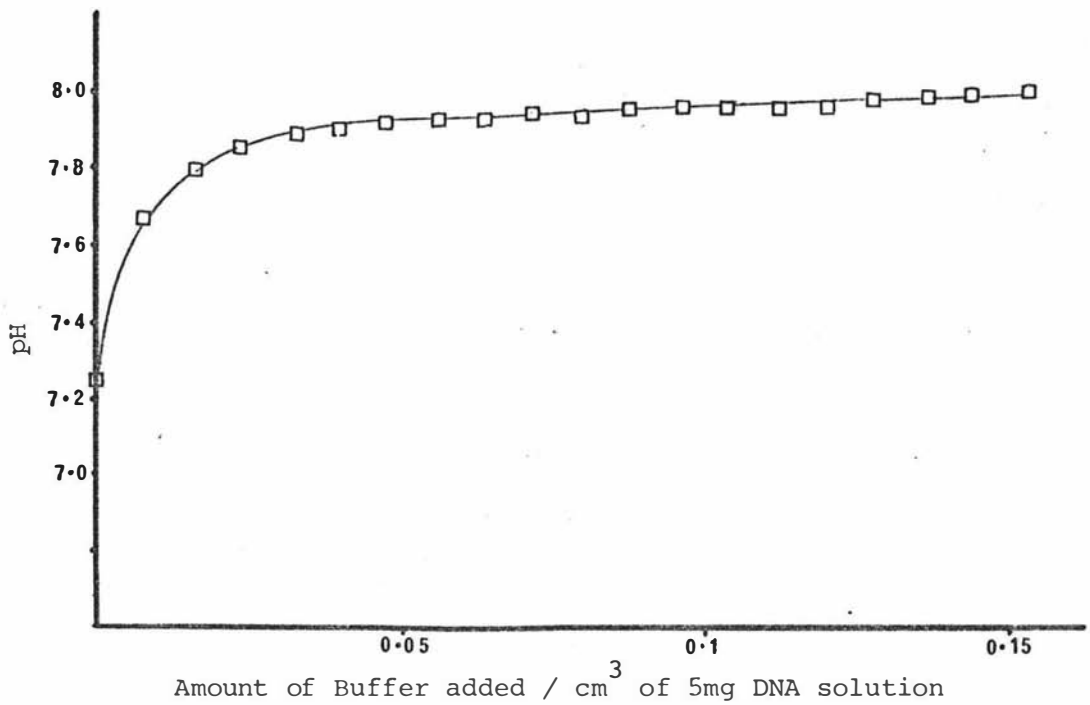


FIGURE THIRTY THREE: The Buffering Capacity if DNA, used to determine the amount of buffer required to ensure a constant pH 8.0 on addition of DNA to Bovine Seminal Plasma.

temperature with 1 hour contact time (FIGURE THIRTY FOUR). 10mg DNA /cm^3 seminal plasma was chosen for use in the general extraction process.

19.1d The Effect of Contact Time on the Adsorption of Antibacterial Activity.

The amount of antibacterial activity adsorbed by DNA from pH 8.0 buffered bovine seminal plasma after 1 hour contact time differed only slightly from that adsorbed when DNA was left a further 18 hours (FIGURE THIRTY FIVE). There was some indication, however, that the increased time allowed closer binding of the active factor to DNA (TABLE XXIII). The washes of the DNA-protein complex formed after 1 hour contact time (10mg DNA/cm^3 seminal plasma) contained substantially more activity (31% of the original antibacterial activity in bovine seminal plasma), than the washes of complexes allowed longer contact time (12.5% of original activity). The increase in the amount of activity adsorbed during 18 hours contact time was not considered sufficient to prolong the extraction process, and, therefore, 1 hour contact time was selected for normal use.

19.1e The Effect of Temperature on the Adsorption of Antibacterial Activity.

Holding the DNA/seminal plasma mixture at 60'C increased the amount of antibacterial activity adsorbed by 10mg DNA/cm^3 seminal plasma to 76.8% of the original activity compared with 56.4% when the adsorption was performed at room temperature (TABLE XXIV). All extractions were therefore carried out at 60'C.

19.1f Recycling Bovine Seminal Plasma after DNA Extraction.

After removal of the DNA-protein complex, the supernatant still contained some antibacterial activity. It was therefore decided to re-treat this solution with fresh DNA to investigate the possibility of recovering additional activity from previously adsorbed seminal plasma. For this experiment it was necessary to dilute the supernatant 1 : 1 with pH 8.0 phosphate buffer, in order to decrease the salinity of the seminal plasma to enable the added DNA to dissolve. A further 36.9% of the remaining activity, or 10.5% of the original activity was adsorbed by the fresh addition of 10mg DNA/cm^3 seminal plasma.

From these studies it was concluded that the optimum conditions for the maximum adsorption of antibacterial activity from bovine seminal plasma by DNA required the use of 10mg of highly polymerised DNA per cm^3 bovine seminal plasma at pH 8.0 . The DNA was left in contact with the seminal plasma for 1 hour at 60'C to permit the formation of complexes between the

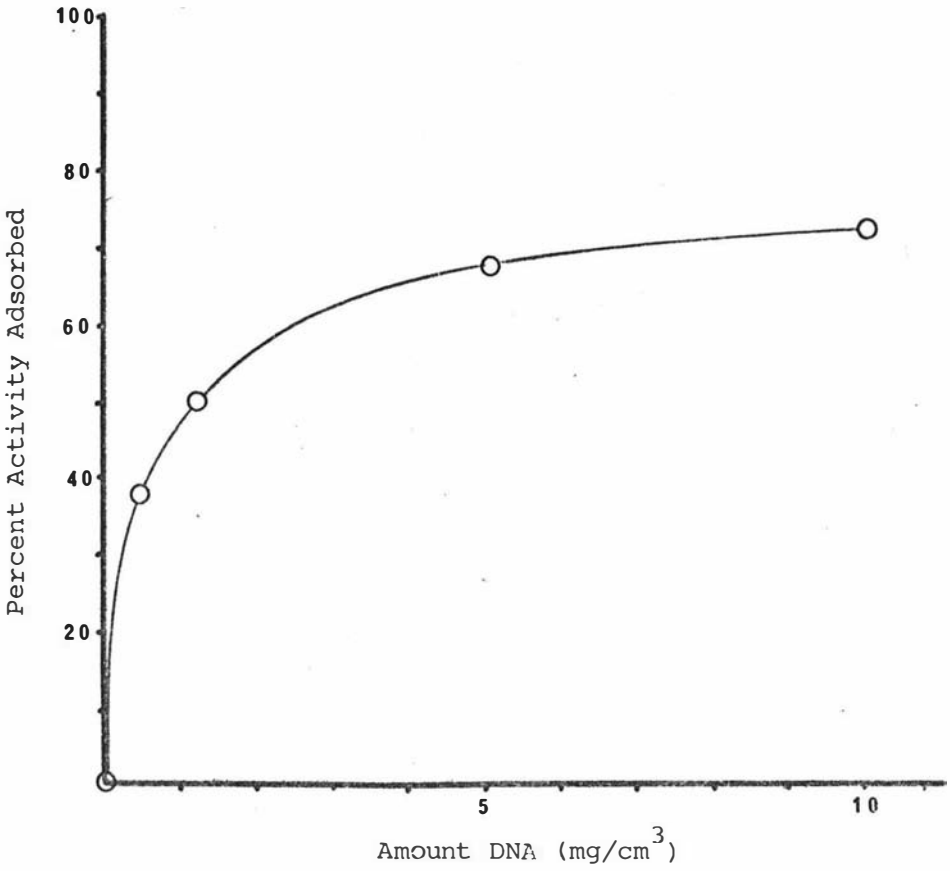


FIGURE THIRTY FOUR: Amount of DNA required for Maximum Adsorption of Antibacterial Activity from pH 8.0 Bovine Seminal Plasma.

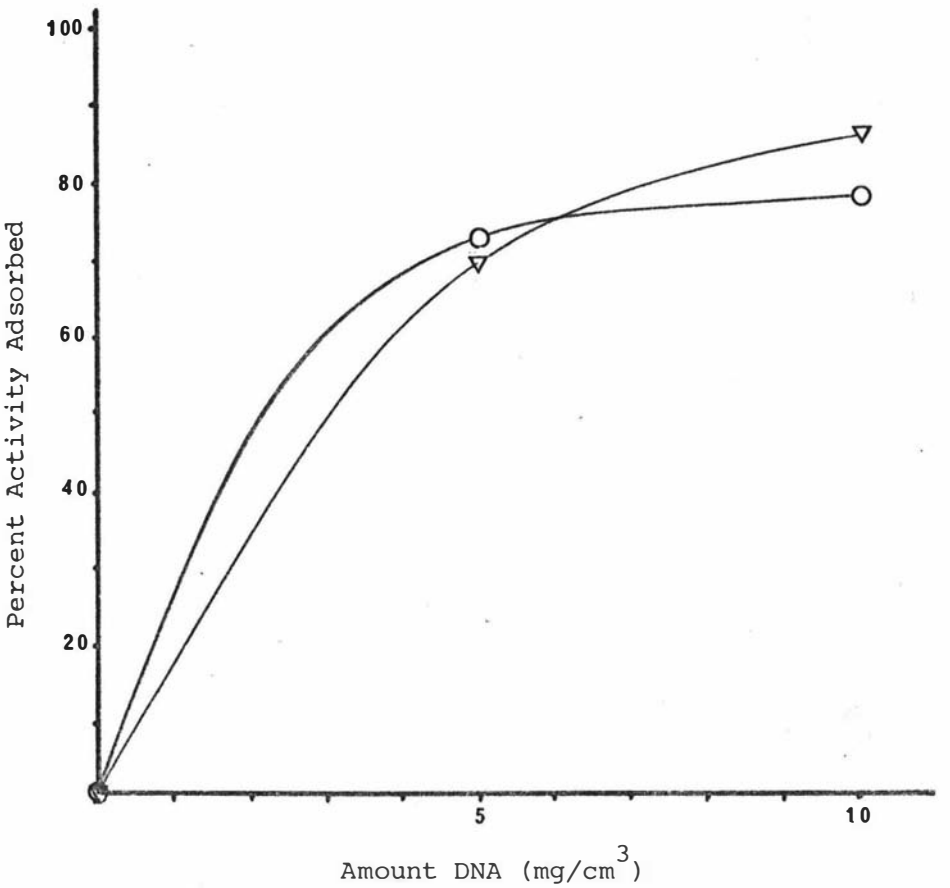


FIGURE THIRTY FIVE: The Effect of Contact Time on the Amount of Antibacterial Activity adsorbed by DNA from Bovine Seminal Plasma .

O= 1 hour contact time
∇= 18 hours contact time

TABLE XXIII.

THE EFFECT OF CONTACT TIME ON ADSORPTION OF ANTIBACTERIAL ACTIVITY FROM BOVINE SEMINAL PLASMA BY DNA.

CONTACT TIME	AMOUNT DNA (mg/cm^3)	ORIGINAL ACTIVITY (LzU_e)	ACTIVITY REMAINING (LzU_e)		ACTIVITY ADSORBED (LzU_e)
1 hour	5	19,310	S/N*	3,875	
			W_1 #	645	
			W_2 ¢	645	
				5,165	14,145 (73.3%)
1 hour	10	19,310	S/N*	2,775	
			W_1 #	1,125	
			W_2 ¢	443	
				4,343	14,967 (77.5%)
18 hours	5	19,190	S/N*	3,875	
			W_1 #	1,350	
			W_2 ¢	443	
				5,668	13,588 (70.5%)
18 hours	10	19,190	S/N*	2,400	
			W_1 #	300	
			W_2 ¢	0	
				2,700	16,490 (85.9%)

FOOTNOTES : * S/N = supernatant after DNA treatment.

\# W_1 = the first wash of the DNA-protein complex.

¢ W_2 = the second wash of the DNA-protein complex.

TABLE XXIV.

THE EFFECT OF HEAT ON THE ADSORPTION OF ANTIBACTERIAL ACTIVITY FROM BOVINE SEMINAL PLASMA BY DNA (10mg/cm^3).

TEMPERATURE	ORIGINAL ACTIVITY (LzU_e)	ACTIVITY REMAINING (LzU_e)	ACTIVITY ADSORBED (LzU_e)
Ambient (25'C)	55,000	24,000	31,000 (56.4%)
Heat (60'C)	55,000	12,750	42,550 (76.8%)

TABLE XXV.

THE DISSOCIATION OF THE DNA-PROTEIN COMPLEX IN THE EXTRACTION OF ANTIMICROBIAL FACTORS FROM BOVINE SEMINAL PLASMA.

METHOD	ORIGINAL ACTIVITY (LzU_e)	ACTIVITY REMAINING (LzU_e)	ACTIVITY ADSORBED (LzU_e)	ACTIVITY RECOVERED (LzU_e)	PERCENT YIELD
NaCl dissociation	76,500	21,000	55,500	7,200	13.0
HCl dissociation	76,500	21,000	55,500	4,375	7.9
Citrate dissociation	76,500	21,000	55,500	42,000	75.7

active factor(s) and the DNA. The DNA-protein complex(es) was harvested by increasing the salinity of the seminal plasma to 0.14M NaCl which decreased the solubility of the DNA-protein complex(es). This complex(es) was washed with 0.14M saline to remove any non-adsorbed seminal plasma proteins , and once clean of contaminating proteins was harvested by centrifugation. The resultant material was treated to dissociate the DNA-protein complex(es) and to recover the active moiety, free of the DNA carrier.

19.2 Conditions for the Dissociation of the DNA-Protein Complex.

Several methods were investigated in attempts to recover the active moiety from its complex(es) with DNA.

19.2a 2M NaCl was used to dissociate the DNA-protein complex (TABLE XXV), followed by precipitation of the free DNA with an equal volume of ethanol. This yielded 13% of the activity calculated to have been adsorbed onto the DNA.

19.2b The DNA-protein complex was dissolved in distilled water and made 0.2N with respect to HCl ; this precipitated the released DNA and the supernatant contained 7.9% of the activity calculated to have been adsorbed onto the DNA.

19.2c 2M Sodium citrate was used to dissociate the complex and then the solution was adjusted to 0.5M citrate to allow assay of the anti-bacterial activity present (CHAPTER 4.4). The released DNA was precipitated with an equal volume of ethanol ; this method yielded 75.7% of the activity calculated to have been adsorbed onto the DNA.

This level of citrate was chosen since increasing citrate concentrations in the original seminal plasma decreased the ability of DNA to complex with the antibacterial factor(s) (FIGURE THIRTY SIX). At 0.5M citrate no activity was adsorbed by DNA; the presence of citrate was thought to have blocked the interaction of DNA with the active moiety and prevented the formation of the DNA-protein complex(es). Because of this it was postulated that this molarity of citrate would also dissociate the complex(es) once it was formed.

Thus, dissociation of the washed DNA-protein complex(es) was best achieved by resuspending the pellet in 0.5M sodium citrate. The released DNA was then precipitated out of solution by the addition of an equal volume of ethanol, and removed by centrifugation at 4000 rpm for 15 minutes. This left the active moiety released from the DNA-protein complex in a citrate/ethanol solution.

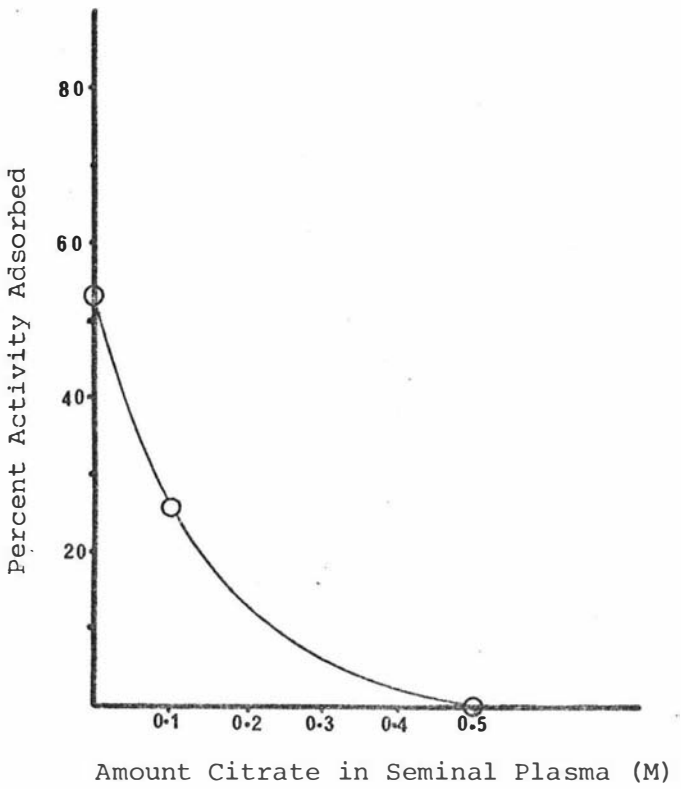


FIGURE THIRTY SIX: The Effect of Citrate in Bovine Seminal Plasma on the Adsorption of Antibacterial Activity by DNA ($10mg/cm^3$).

19.3 Recovery of the Active Fraction from the Citrate/Ethanol Solution.

Several methods were tested for the recovery of the antimicrobial factor from the citrate/ethanol solution after dissociation of the DNA-protein complex, and removal of the free DNA.

19.3a Ammonium sulphate precipitation at 20, 30, 45, 50, 55, 60 and 70% levels precipitated very little of the antimicrobial activity present in solution.

19.3b Acetone precipitation (6 volumes acetone : 1 volume active solution) in slightly acid conditions brought down some of the activity, although some activity was still present in the supernatant.

19.3c If the citrate/ethanol solution was held below 0'C, some of the citrate crystallised out and could be removed by centrifugation in the cold (0'C) at 10,000rpm for 15 minutes. The antibacterial activity in the supernatant could be precipitated out in a relatively pure form by the addition of increasing amounts of ethanol. A further 1 : 1 dilution with ethanol gave a large precipitate which was removed by a further centrifugation, but which contained little antibacterial activity. The next addition of an equal volume of ethanol (87.5% ethanol v/v) to the resultant supernatant yielded a precipitate with high antibacterial activity ;little or no activity remained in the supernatant. This completed procedure represented a 1 : 7 dilution with ethanol of the original citrated solution.

19.4 Summary of the Extraction Process for the Isolation of an Antibacterial Factor from Bovine Seminal Plasma by Adsorption onto DNA.

The final extraction process adopted to isolate the factor responsible for at least some of the antimicrobial activity present in bovine seminal plasma is summarised in FIGURE THIRTY SEVEN.

There were some minor variations in successive extraction runs. The amount of activity adsorbed onto the DNA ranged from 53.4% to 76.7% of the total activity present in bovine seminal plasma. Often the amount of activity removed from the DNA-protein complex was greater than the amount of activity calculated to have been adsorbed. This may have been due to an enhancement of the activity of the factor(s) in the absence of other seminal plasma constituents.

A representative calculation associated with the extraction of antimicrobial compounds from bovine seminal plasma is shown in TABLE XXVI.

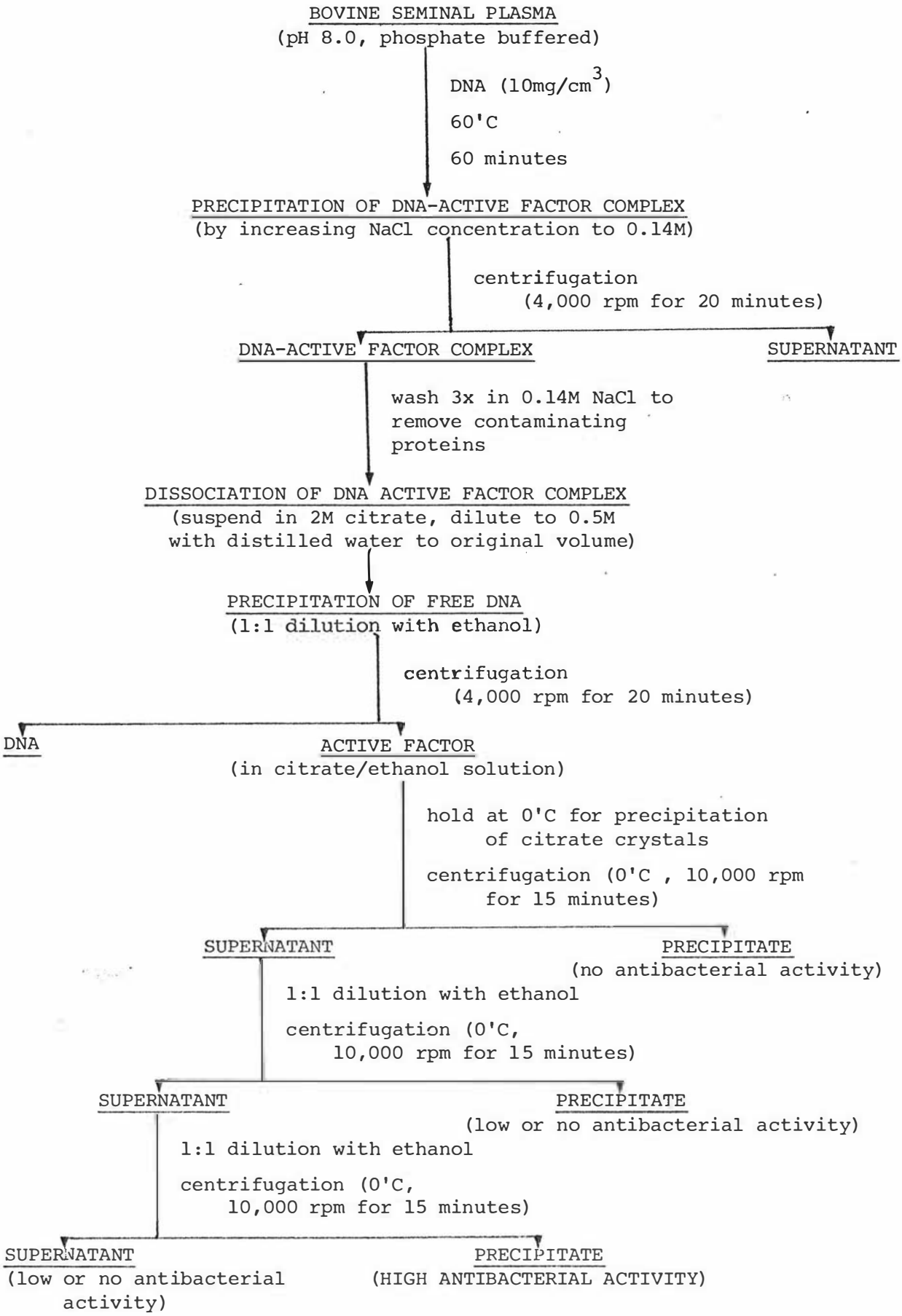


FIGURE THIRTY SEVEN: Format for the isolation of an antibacterial factor from bovine seminal plasma by adsorption onto DNA.

TABLE XXVI.

CALCULATIONS OF THE AMOUNT OF ANTIBACTERIAL ACTIVITY HARVESTED FROM BOVINE SEMINAL PLASMA DURING A RUN OF THE DNA EXTRACTION PROCESS.

	ACTIVITY (LzU_e/cm^3)	VOLUME (cm^3)	TOTAL (LzU_e)
ACTIVITY IN SEMINAL PLASMA	18,250	36.00	657,000
Activity in Supernatant after DNA treatment	7,500	16.50	123,750
Activity in Wash 1	2,900	15.75	45,675
Activity in Wash 2	1,900	17.75	33,725
Activity in Wash 3	1,025	21.00	21,525
ACTIVITY REMAINING IN SUPERNATANT AND WASHES			224,675
ACTIVITY ADSORBED BY DNA	657,000 - 224,675		= 432,325 (66%)

	ACTIVITY (LzU_e/mg)	WEIGHT (mg)	TOTAL (LzU_e)
Amount recovered in			
1 : 1 precipitate	0	153.6	0
1 : 3 precipitate	86	2,928.8	251,877
1 : 7 precipitate	2,250	234.8	528,300
TOTAL AMOUNT RECOVERED FROM DNA			780,177 (180%)

NB : The Total Activity Recovered (780,177 LzU_e) was greater than that calculated as having been adsorbed by the DNA from the original bovine seminal plasma (432,325 LzU_e).

CHAPTER 20: CHARACTERISATION OF THE ANTIMICROBIAL FACTOR ISOLATED FROM BOVINE SEMINAL PLASMA BY DNA ADSORPTION.

PART A : BIOLOGICAL PROPERTIES :

20.1 Antimicrobial Activity.

The material eluted from DNA was tested by the radial diffusion assay (CHAPTER 4.3) ; it inhibited M.lysodeikticus, E. coli, S. aureus, and a Bacillus sp.[1], but had no effect on P. vulgaris ; this latter strain was also resistant to whole bovine seminal plasma. Several Mycoplasma spp. , M. hominis (2 strains), M. arthritidis, M. ovipneumoniae, were also inhibited by the isolated antimicrobial factor (CHAPTER 8.3). This spectrum of activity was similar to that of the heat stable activity observed with whole bovine seminal plasma (TABLE XXVII). It has been concluded that the factor isolated from bovine seminal plasma by adsorption onto DNA is a significant component of the heat stable activity seen in whole bovine seminal plasma.

It was also demonstrated that the extracted material exerted a rapid bacteriocidal effect on M. lysodeikticus, E.coli and Ps.fluorescens (CHAPTER 17.2 , FIGURE TWENTY SEVEN).

20.2 The Specific Activity of DNA-isolated Material.

The protein content of whole bovine seminal plasma and DNA-isolated material was measured according to the method of Lowry et al (1951) using egg white lysozyme as the reference protein (CHAPTER 10.2).

The DNA-isolated antimicrobial factor had 15x higher specific activity (8.0×10^3 LzU_e/mg protein) than the original bovine seminal plasma (5.24×10^2 LzU_e/mg protein).

PART B : PHYSICO-CHEMICAL PROPERTIES :

20.3 The Effect of Trypsin Treatment.

DNA-isolated material lost 60% of its activity when exposed to trypsin for 30 minutes at 30'C (CHAPTER 7.2) , see TABLE XXVIII. This is a proportionally lower loss than that sustained by whole seminal plasma after similar treatment (TABLE IX).

20.4 The Effect of Periodate Treatment.

The antibacterial activity of DNA-isolated material was not affected by periodate treatment (CHAPTER 7.3), see TABLE XXVIII. This agrees with

FOOTNOTE : [1] bacterium isolated by Shannon et al (1974).

TABLE XXVII.

COMPARISON OF THE SPECTRUM OF ACTIVITY OF DNA-ISOLATED MATERIAL WITH THAT OF WHOLE BOVINE SEMINAL PLASMA.

TEST ORGANISM	ACTIVITY (LzU /mg protein) IN : BOVINE SEMINAL PLASMA	HEATED BOVINE SEMINAL PLASMA (60 minutes at 60'C)	DNA ISOLATED MATERIAL
Micrococcus lysodeikticus	1,462	1,462	5,375
Escherichia coli	173	173	1,800
Proteus vulgaris	0	0	0
Staphylococcus aureus	14	0	0
Staphylococcus aureus	274	0	1,800
Bacillus subtilis	25	0	slight
Bacillus sp.*	216	216	3,813
Mycoplasma hominis	14,231	10,385	4,750
Mycoplasma arthritidis	6,538	5,385	5,375
Mycoplasma ovipneumoniae	1,971	1,558	2,350

FOOTNOTE : * bacterium isolated by Shannon et al (1974).

TABLE XXVIII.

THE EFFECT OF TRYPSIN TREATMENT, PERIODATE TREATMENT AND HEAT ON THE ANTIBACTERIAL ACTIVITY OF MATERIAL ISOLATED FROM BOVINE SEMINAL PLASMA ADSORPTION ONTO DNA. (5mg isolate/cm^3).

TREATMENT	ACTIVITY (LzU e/cm^3) before treatment	ACTIVITY (LzU e/cm^3) after treatment	PERCENT ACTIVITY LOST BY TREATMENT
Trypsin	1,175	470	60
Periodate	1,330	1,330	0
Heat (60'C for 60 minutes)	1,330	1,330	0

TABLE XXIX.

THE ABSORBANCE VALUES FOR BOVINE SEMINAL PLASMA AND DNA-ISOLATED ANTIMICROBIAL FACTOR.

MATERIAL	ABSORBANCE (230nm)	ABSORBANCE (280nm)	RATIO 280/230
Bovine seminal plasma	23.20	5.00	0.22
DNA extract	1.84	0.52	0.28

results obtained with whole bovine seminal plasma (TABLE X).

20.5 The Effect of Heat.

During the extraction process (CHAPTER 19.4) the bovine seminal plasma was held at 60'C for 60 minutes while the DNA was in contact with the bovine seminal plasma. The isolated material retained its activity at this temperature both during this process and when it was heated to 60'C for 60 minutes after the isolation procedure was completed (TABLE XXVIII).

20.6 The Absorbance Spectrum of Bovine Seminal Plasma and DNA-isolated Antimicrobial Factor.

Bovine seminal plasma and DNA-isolated material in citrate/ethanol solution were both diluted ($0.2cm^3$ / $3.0cm^3$) with distilled water and their absorbances measured at 230nm and 280nm (TABLE XXIX). The ratio of absorbance at 280nm over that at 230nm gave some indication of the number of aromatic residues per peptide bond. For the DNA-isolated factor this ratio was greater than that for whole bovine seminal plasma, indicating that there had been an increase in the relative number of aromatic residues present in the isolated material compared with the original seminal plasma proteins.

20.7 Ultracentrifugation of the DNA-isolated Material.

DNA-isolated material ($100mg/cm^3$) was dialysed against 0.2M phosphate buffer for 3 hours before its sedimentation rate was examined in a Beckman Model E Analytical Ultracentrifuge.

DNA-isolated material was found to occur as two separable molecular weight species with different sedimentation velocities. PLATE TWO is a representative illustration of the two detectable peaks corresponding to these two molecular weight species. Their delineation was not complete and during the investigation no clear separation of the peaks occurred, although the calculated sedimentation velocities were noticeably different. This type of sedimentation is commonly recorded for proteins which reversibly polymerise due to self association (Colowick & Kaplan 1973).

The distances moved by each molecular species are given in FIGURE THIRTY EIGHT. From this it was possible to calculate the sedimentation coefficients of the two components using the equation

$$s = \frac{d \log_n x}{d t} \cdot \frac{1}{w^2}$$

where the average rotor velocity was 55822 rpm, giving an angular

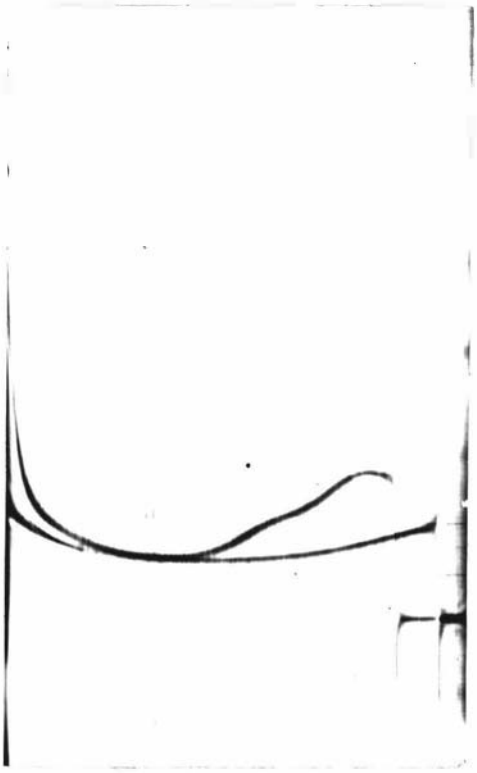


PLATE TWO : Ultracentrifugation (55822 rpm) of Material isolated from Bovine Seminal Plasma by Adsorption onto DNA, showing two distinct protein species with differing sedimentation velocities.

fast moving peak , S = 3.70
slow moving peak , S = 0.94

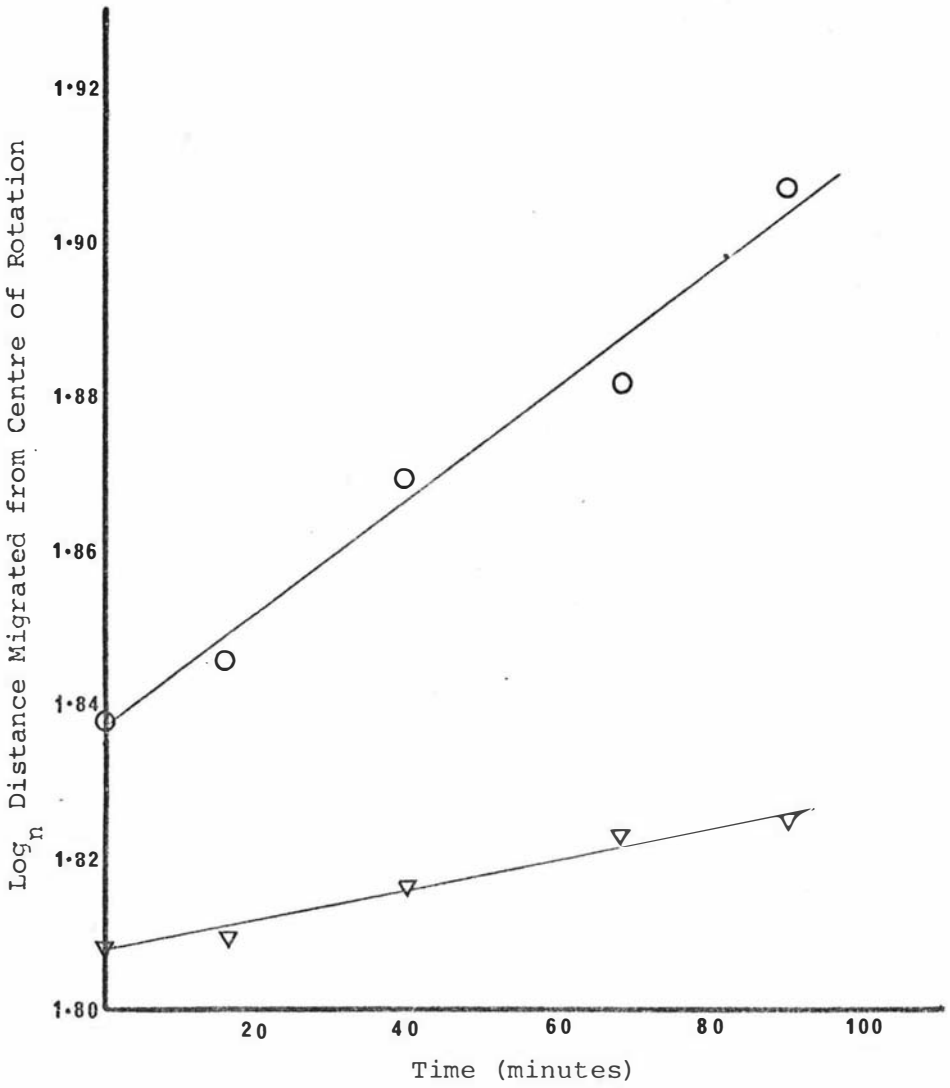


FIGURE THIRTY EIGHT: Sedimentation Rates under Ultracentrifugation (55822 rpm) of Two Molecular Species present in Material isolated from Bovine Seminal Plasma by Adsorption onto DNA.

velocity, w , of 5846 radians per sec.

For the slow sedimenting species, the sedimentation rate (i.e. the slope of the line in FIGURE THIRTY EIGHT)

$$\frac{d \log_n x}{d t} \quad \text{was} \quad 3.2 \times 10^{-6}$$

therefore

$$s = \frac{3.2 \times 10^{-6}}{5846 \times 5846} \quad \text{or} \quad 0.94 \times 10^{-13} \quad \text{sec.}$$

For the fast sedimenting species, the sedimentation rate was 1.25×10^{-5} , and therefore $s = 3.7 \times 10^{-13}$ sec.

S values of approximately 0.94×10^{-13} , represent proteins of molecular weight of 6,500 (bovine pancreas trypsin inhibitor) to 10,400 (tetrahymena proteinase). S values of 3.7×10^{-13} represent proteins with molecular weights of 33,600 (bovine thrombin) to 74,000 (phospho-gluco-mutase), see Sober (1970).

The data quoted in this study were obtained at the highest resolution of the equipment which was available. Back diffusion of the sedimenting molecules may have led to slower apparent sedimentation rates than the true values. Thus, the molecular weight indications were regarded as an approximate guide only. The most important information gained from the ultracentrifugation study was that the antimicrobial factor, isolated from bovine seminal plasma by adsorption onto DNA, existed in at least two forms, sedimenting in a manner characteristic of self-associating protein species.

20.8 Native Polyacrylamide Gel Electrophoresis.

The migratory properties of DNA-isolated antimicrobial activity in native polyacrylamide gel electrophoresis (CHAPTER 7.5) varied considerably with changes in the ionic environment and the microsolute species with which it was associated.

Native polyacrylamide gel electrophoresis at pH 8.7 of DNA-isolated material in the citrate/ethanol solution obtained in the extraction procedure, yielded a smear of cationic material with no well defined banding pattern (FIGURE THIRTY NINE (i)). This solution contained a large amount of the citrate used to strip the active factor off the DNA, and possibly some NaCl from the solution used to wash the DNA-protein complex before the stripping process. Removal of these electrolytes and solute molecules was possible by discontinuous diafiltration ; this washed out solute molecules while retaining the proteins (CHAPTER 10.3). The

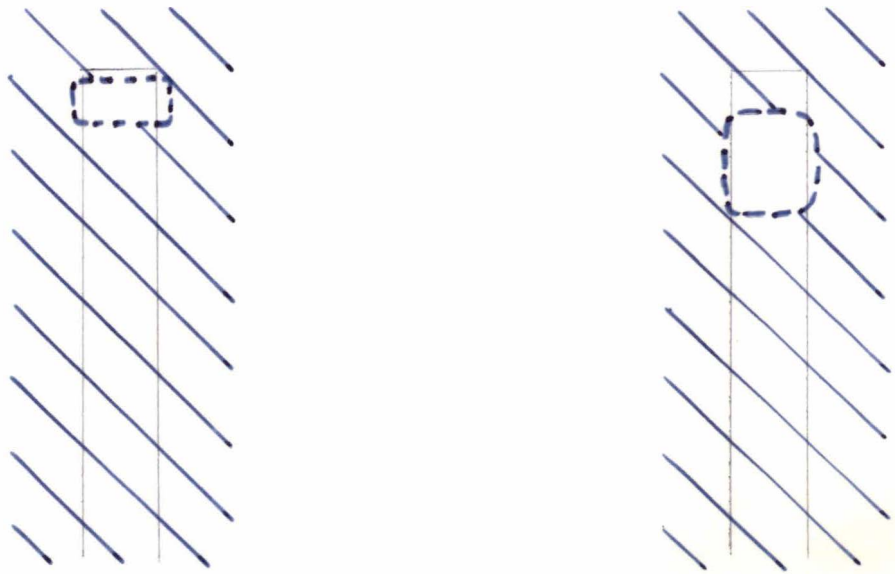


FIGURE THIRTY NINE (a): Inhibition Zones ( ) produced by Electrophoresed Cationic Proteins from DNA-isolated Material (CHAPTER 19.4) after pH 8.6 Native Polyacrylamide Gel was overlaid with agar seeded with Micrococcus lysodeikticus.

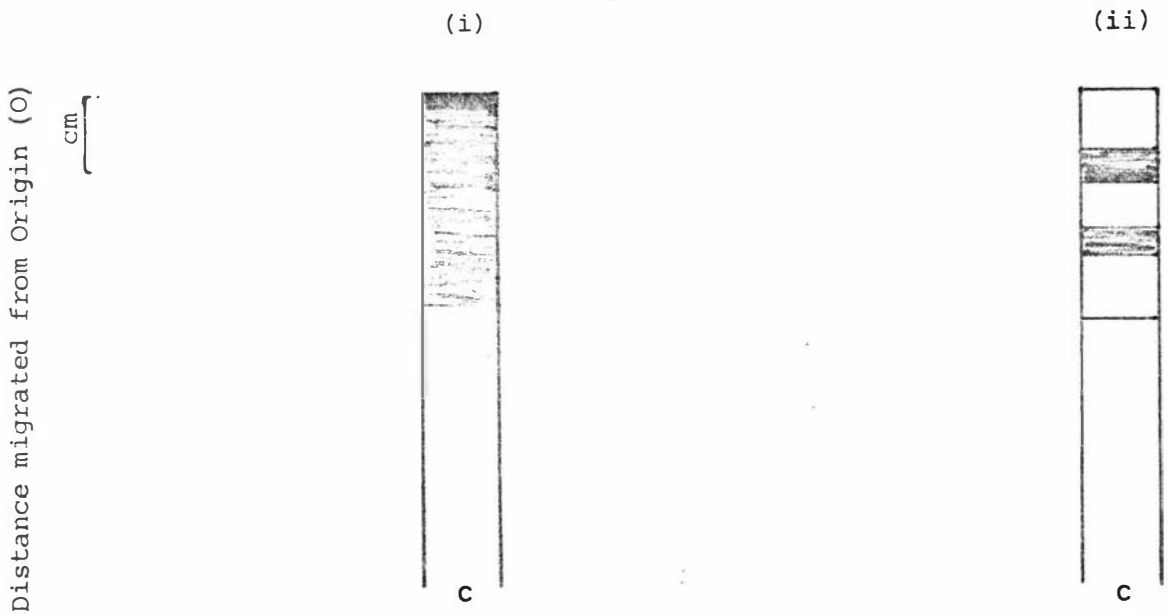


FIGURE THIRTY NINE (b): Electrophoretic Characteristics (Native Polyacrylamide Gel Electrophoresis, pH 8.6) of Cationic Proteins in Material Isolated from Bovine Seminal Plasma by Adsorption onto DNA.

(i) = DNA-isolated Material in Citrate/Ethanol Solution.

(ii)= DNA-isolated Material after Diafiltration to remove Citrate .

C = Cationic Proteins

solution behind the membrane retained its antibacterial activity throughout the diafiltration process.

The amounts of citrate present in the effluents and the final solution after diafiltration were measured by the colorimetric procedure (McArdle 1955) described in CHAPTER 10.4 . The first two effluents by the diafiltration process contained more than 0.5mg citrate/cm^3 ; the third effluent contained 0.11mg citrate/cm^3 , and the solution behind the membrane (i.e. the active material) contained 0.37mg citrate/cm^3 . Thus, some of the citrate remained bound to the DNA-isolated material since the concentration of citrate present in the diafiltered material was greater than that in the final wash effluent.

The diafiltered material showed two distinct cationic bands on native polyacrylamide gel electrophoresis (FIGURE THIRTY NINE (ii)).

DNA-isolated material which had been precipitated with ethanol and lyophilised showed little cationic material on native polyacrylamide gel electrophoresis (pH 8.7), and considerable smearing of anionic material with little banding patterns (PLATE THREE (i)). The difference between this material and that described above is thought to be due to the effect of higher ethanol concentrations on the conformation of the protein molecule and therefore on its ability to interact with the citrate solution present in the precipitated material, and on its electrophoretic properties.

Dialysis of the precipitated DNA-isolated material against 0.2M phosphate buffer resulted in the production of a single anionic band at pH 8.7 . No protein was present in the dialysate (PLATE THREE(ii)). With repeated handling (thawing and refreezing) over a period of three weeks, other protein bands were generated. Eventually this sample of DNA-isolated material produced three anionic bands and one cationic band by native polyacrylamide electrophoresis at pH 8.7 (PLATE THREE (iii)) .

Further variations in native polyacrylamide gel electrophoretic properties were shown after different treatments of the dialysed DNA-isolated material (PLATE FOUR):

(a) heating to 60'C for 60 minutes had little effect on the properties of the dialysed material, although there may have been some increase in the slower moving anionic band (PLATE FOUR (ii)).

(b) diafiltration of the dialysed material, by diluting with buffer and then reducing the solution to its original volume behind a UM 2 ultrafilter (CHAPTER 10.3) resulted in a return to the totally anionic situation achieved after dialysis of the ethanol precipitated material off

PLATE THREE: Native Polyacrylamide Gel Electrophoresis (pH 8.7) of DNA-isolated Antimicrobial Material from Bovine Seminal Plasma.

(i) Ethanol Precipitate

(ii) Dialysate from Dialysis of Ethanol Precipitate against 0.2M pH 8.0 phosphate buffer

(iii) Ethanol Precipitate after dialysis against 0.2M pH 8.0 phosphate buffer : Changes occurring over a period of 3 weeks after repeated thawing and refreezing of the preparation.

A = Anionic Proteins
C = Cationic Proteins.

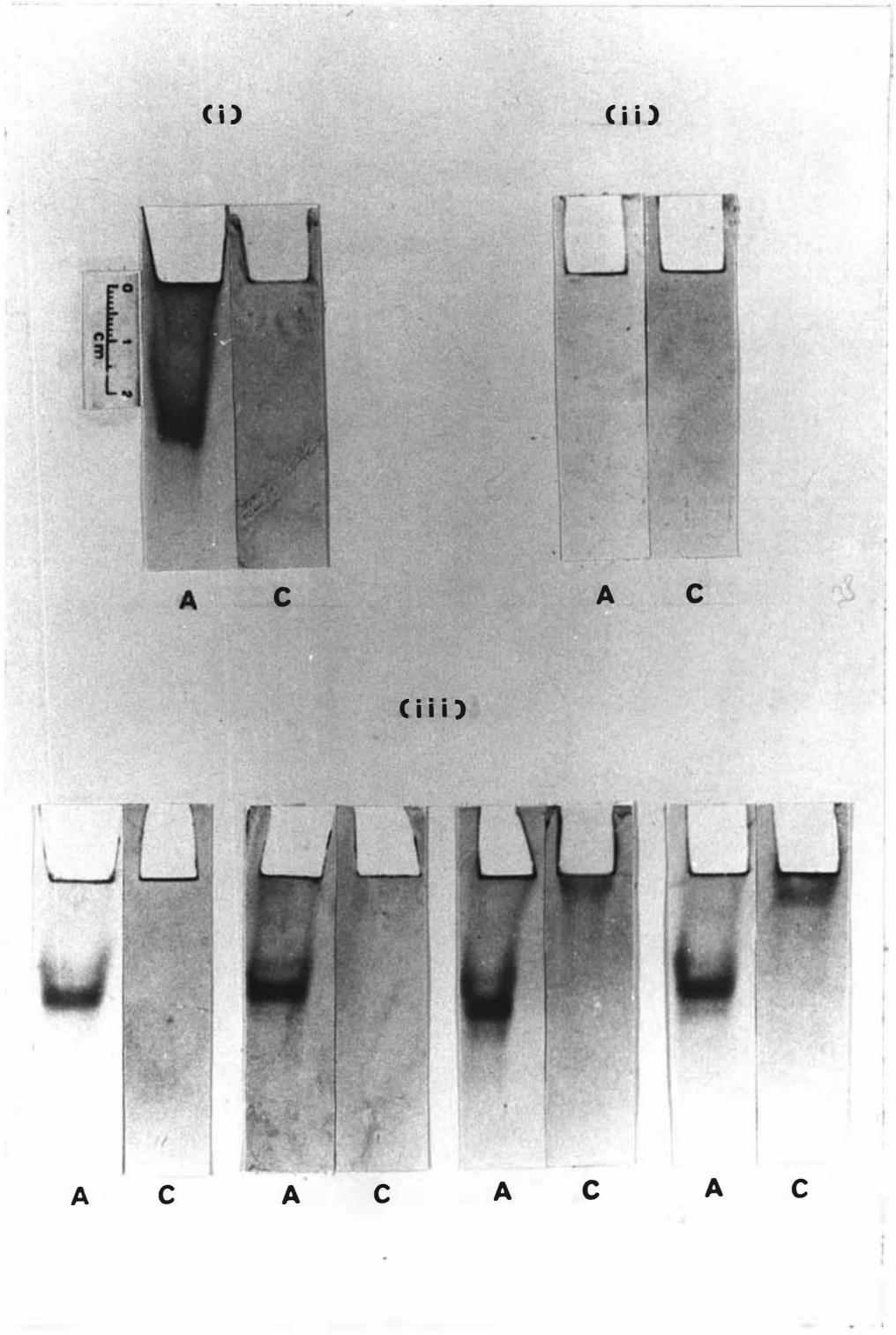
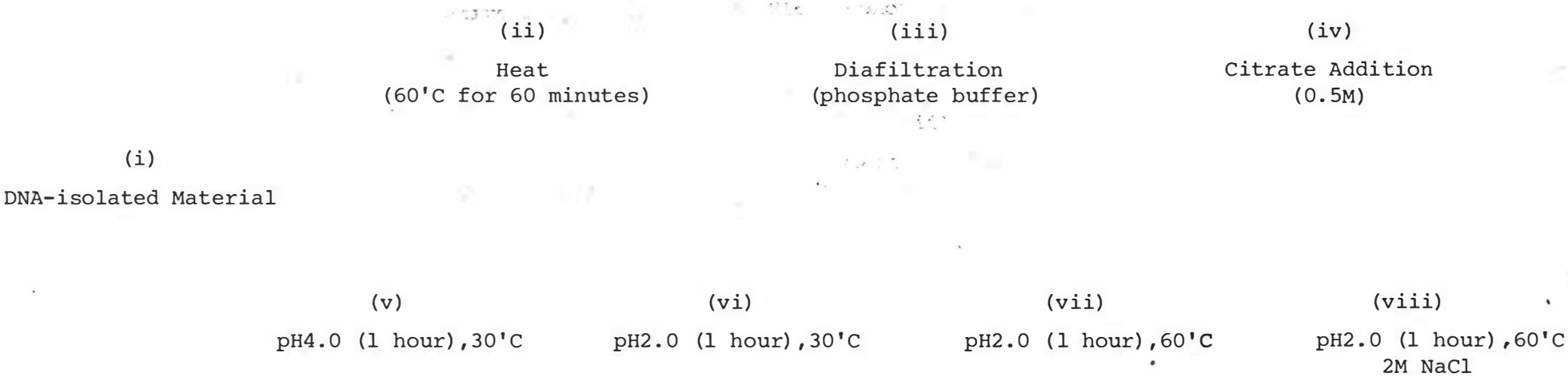
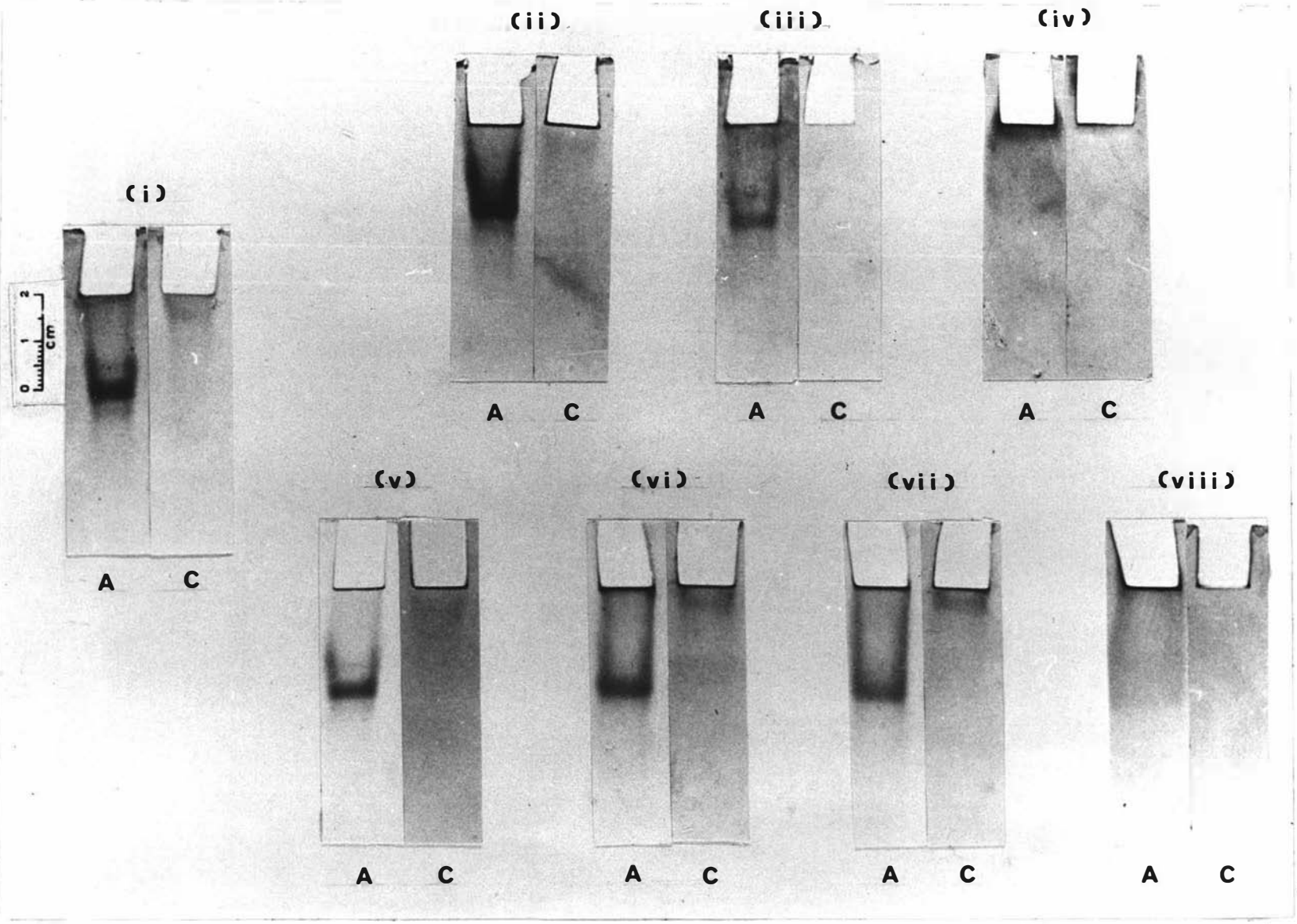

(i)
(ii)
0
1
2
cm
A
C
A
C
(iii)
A
C
A
C
A
C
A
C

PLATE FOUR : Alterations in the Native Polyacrylamide Gel Electrophoretic Properties of Phosphate Dialysed DNA-isolated Antimicrobial Material.



A = Anionic Proteins
C = Cationic Proteins.


(i)
0 1 2
cm
A C
(ii)
A C
(iii)
A C
(iv)
A C
(v)
A C
(vi)
A C
(vii)
A C
(viii)
A C

the DNA (PLATE FOUR (iii)).

(c) citrate (0.5M) addition markedly reduced the ability of the antimicrobial factor to migrate in native polyacrylamide gels, some protein could be located at or near the origin but little appeared to migrate further into the gel. Citrate presence therefore appeared to neutralise the original charge on the phosphate-protein complexes (PLATE FOUR (iv)).

(d) lowering the pH of the solution of DNA-isolated material in phosphate buffer to pH 2.0 or pH 4.0, holding these acid conditions for 1 hour at 30'C or 60'C , and then neutralising back to pH 8.5 , resulted in the generation of more cationic material from the complexes. The presence of NaCl (2M) during this treatment (pH 2.0 and 60'C) resulted in little protein being visible on electrophoresis. (PLATE FOUR (v) , (vi), (vii) and (viii).

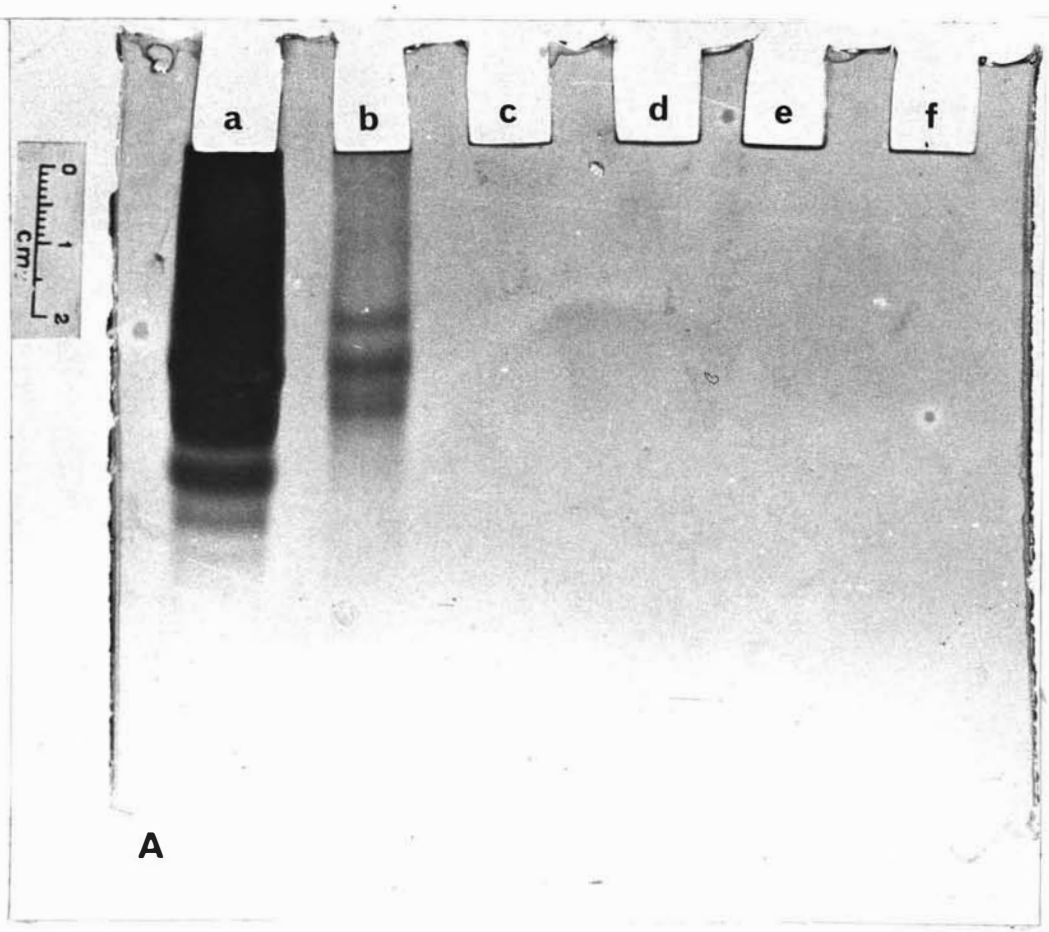
From these results it seems likely that the physical nature of the antimicrobial factor isolated from bovine seminal plasma by adsorption onto DNA alters significantly with the ionic environment and the molecular species with which it is associated.

Comparison of the patterns of native polyacrylamide gel electrophoresis of bovine seminal plasma, phosphate dialysed DNA-isolated antimicrobial factor, lysozyme, polylysine, salmine and spermidine show that little similarities existed between these antimicrobial agents. The active factor isolated from bovine seminal plasma by adsorption onto DNA had no resemblance in native polyacrylamide gel electrophoresis to the selected antimicrobial agents tested (PLATE FIVE).

20.9 SDS Polyacrylamide Gel Electrophoresis of DNA-isolated Material.

The multiple banding seen in native poylacrylamide gel electrophoresis and the two molecular species with different sedimentation coefficients during ultracentrifugation caused doubts as to the actual purity of the material isolated from bovine seminal plasma by adsorption onto DNA. These queries were, however, answered by the appearance of a single protein band during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) which, by adsorbing to protein molecules, neutralises the charge differences caused by amino acid variations, enabling the molecules to migrate solely on the basis of size during electrophoresis.

Using the method described in CHAPTER 10.5 , the antimicrobial factor isolated from bovine seminal plasma by adsorption onto DNA migrated as a single protein band in two separate runs (FIGURE FORTY) ; this



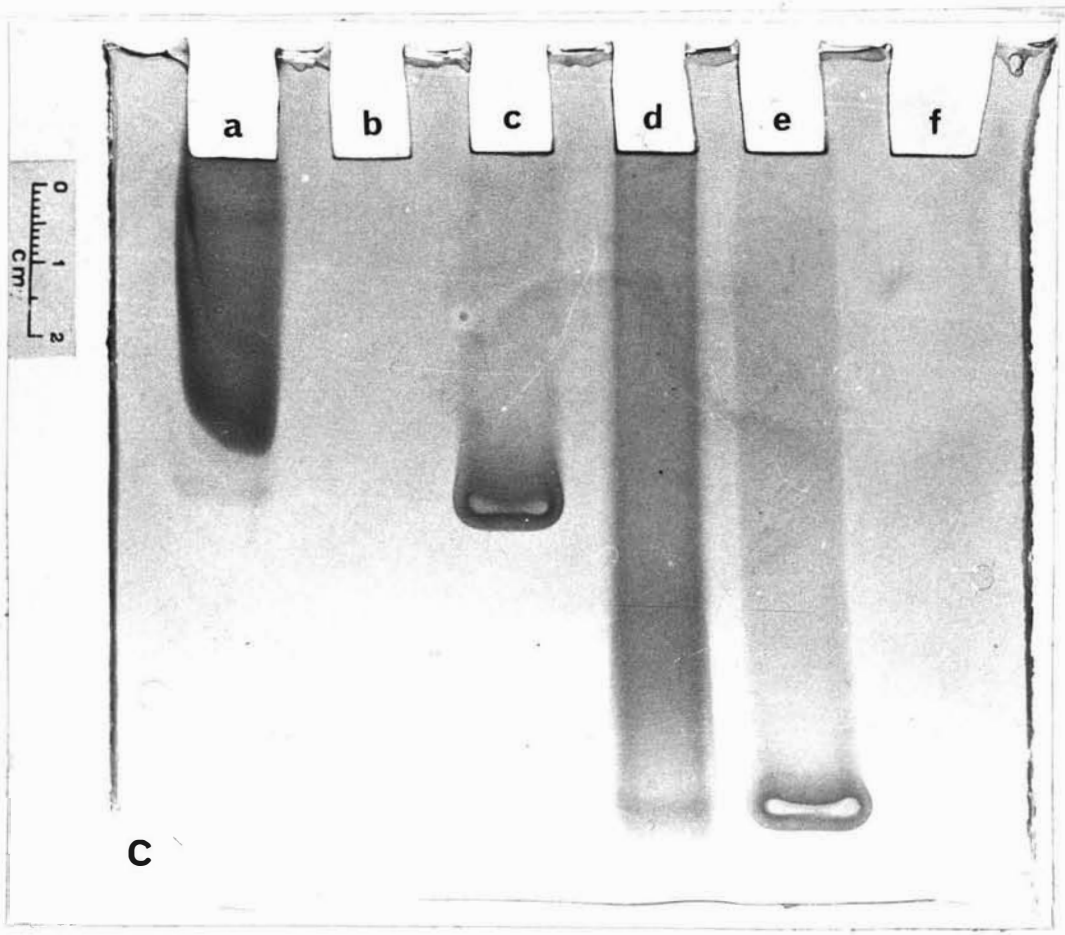


PLATE FIVE : Native Polyacrylamide Gel Electrophoresis (pH 8.6) of : (a) bovine seminal plasma
(b) DNA-isolated material
(c) lysozyme
(d) polylysine
(e) salmine
(f) spermidine

A = Anionic Proteins
C = Cationic Proteins.

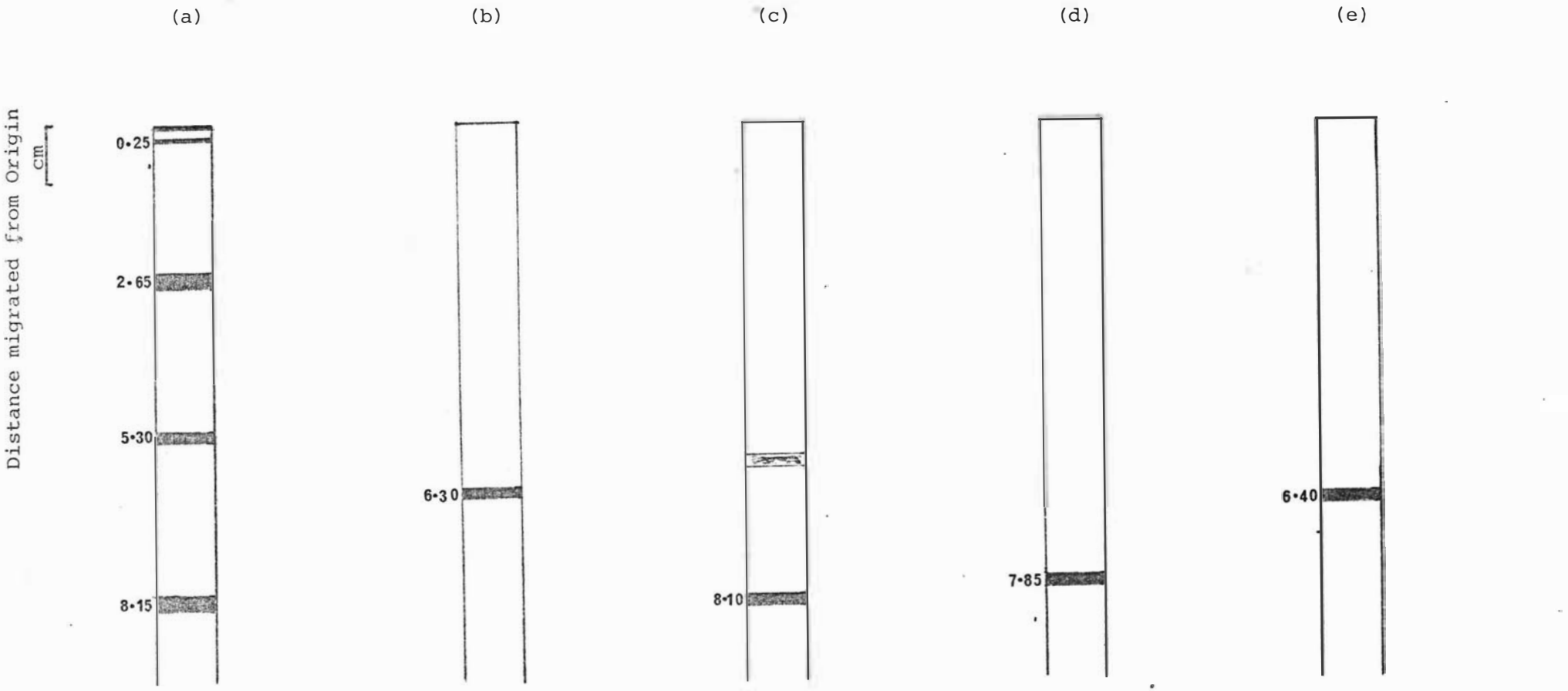


FIGURE FORTY : SDS Polyacrylamide Gel Electrophoresis for the Determination of the Molecular weight of the Antibacterial Factor isolated from Bovine Seminal Plasma by Adsorption onto DNA.

(a) Bovine serum albumin (molecular weight = 136,000 and 68,000) ; Lactic dehydrogenase (molecular weight = 35,000) Myoglobin (molecular weight 17,200).
(b) Trypsin (molecular weight 23,300).
(c) Haemoglobin (molecular weight 68,000 and 15,500).
(d) Lysozyme (molecular weight 14,300).
(e) DNAisolated material.

indicated that by eliminating the charge effects and dissociating aggregated forms, the DNA-isolated material behaved as a single molecular weight species which migrated at a rate consistent with a molecular weight of 24,550 to 25,700 daltons (FIGURE FORTY ONE). This gave an average molecular weight calculation of 25,100 daltons.

20.10 Amino Acid Composition of the DNA-isolated Material.

The relative amino acid composition of DNA-isolated antimicrobial material from bovine seminal plasma and several lysozymes are listed in TABLE XXX.

The most notable feature of the amino acid composition of DNA-isolated antimicrobial material from bovine seminal plasma is the lack of arginine residues, since cationic bands often occurred at pH 8.6 in native polyacrylamide gel electrophoresis. However, the lysine and histidine residues are numerous and amide derivatives of glutamic and aspartic acids may contribute to the basic nature of the protein.

The factor responsible for some of the antimicrobial activity in bovine seminal plasma differed substantially in its overall amino acid composition from that of lysozyme : it had higher numbers of histidine and the aromatic amino acids (phenylalanine and tyrosine) and no arginine moieties.

There is a similar higher number of aromatic amino acids, especially phenylalanine when the amino acid composition of the DNA-isolated factor is compared with the overall protein content of bovine seminal plasma (TABLE XXXI). Arginine is absent from the isolated material but the lysine content of this material is substantially greater than that of whole bovine seminal plasma.

Amino acid analysis also yielded glutamic acid or its amide derivative as the sole N terminal residue in the antimicrobial factor isolated from bovine seminal plasma, indicating the presence of a single protein or the unlikely presence of contaminating proteins with the same N terminal amino acid.

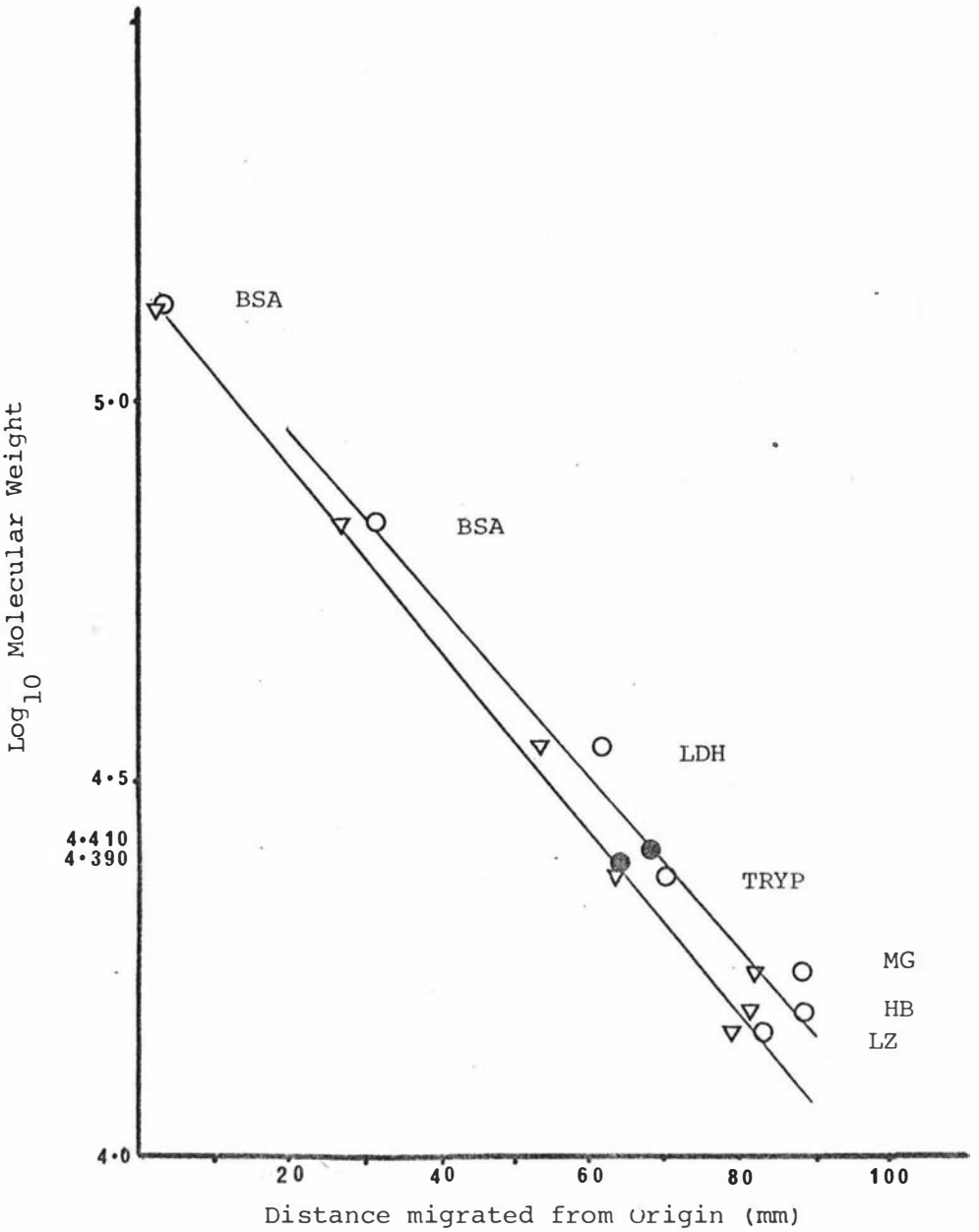


FIGURE FORTY ONE: Molecular weight Determinations of the Antimicrobial Factor isolated from Bovine Seminal Plasma by Adsorption onto DNA, by SDS Polyacrylamide Gel Electrophoresis.

RUN 1 : (▽) regression line , y =426.6 - 82.7x

RUN 2 : (○) regression line , y =454.0 - 87.6x

BSA = bovine serum albumin MG = myoglobin

LDH = lactic dehydrogenase HB = haemoglobin

TRYP= trypsin LZ = egg white lysozyme

●= DNA-isolated Material

TABLE XXX.

PERCENT AMINO ACID COMPOSITION OF DNA-ISOLATED ANTIMICROBIAL FACTOR FROM BOVINE SEMINAL PLASMA compared with those of SEVERAL FORMS OF LYSOZYME.

AMINO ACID	DNA-ISOLATED MATERIAL	HUMAN MILK LYSOZYME*	EGG WHITE LYSOZYME*	GOOSE LYSOZYME*
Glycine	8.2	8.5	9.3	11.0
Alanine	7.0	10.8	9.3	8.3
Valine	4.4	6.9	4.7	5.5
Isoleucine	2.5	3.9	4.7	6.1
Leucine	7.0	6.2	6.2	3.9
Methionine	1.3	1.5	1.6	1.7
Proline	4.4	1.5	1.6	2.8
Cysteine	5.7	6.2	6.2	2.2
Serine	7.6	4.6	7.8	5.0
Threonine	5.7	3.9	5.4	7.2
Aspartic acid	12.7	13.9	16.3	11.0
Glutamic acid	9.5	6.9	3.9	8.3
Lysine	9.5	3.9	4.7	9.9
Histidine	1.9	0.8	0.8	2.8
Arginine	0	10.8	8.5	6.1
Phenylalanine	6.3	1.5	2.3	1.7
Tyrosine	6.3	4.6	2.3	5.0
Tryptophane	-	3.9	4.7	1.7
Number of residues	237	130	129	181
Molecular weight	25,100	14,500	14,300	19,500 to 21,500
N terminal amino acid	glutamic acid	lysine	lysine	arginine

FOOTNOTE : * amino acid composition of lysozymes from Osserman et al (1974).

TABLE XXXI.

AMINO ACID COMPOSITION OF DNA-ISOLATED ANTIMICROBIAL FACTOR AND WHOLE BOVINE SEMINAL PLASMA.

(Methionine as 1, integers to the nearest 0.5)

AMINO ACID	DNA-ISOLATED MATERIAL	BOVINE SEMINAL PLASMA*
Glycine	6.5	-
Alanine	5.5	-
Valine	3.5	2.0
Isoleucine	2.0	2.0
Leucine	5.5	2.5
Methionine	1.0	1.0
Proline	3.5	-
Cysteine	4.5	-
Serine	6.0	-
Threonine	4.5	2.0
Aspartic acid	10.0	-
Glutamic acid	7.5	5.0
Lysine	7.5	3.0
Histidine	1.5	1.5
Arginine	0.0	5.0
Phenylalanine	5.0	2.0
Tyrosine	5.0	-
Tryptophane	-	1.5

FOOTNOTE : * amino acid composition of bovine seminal plasma from Mann (1964).

DISCUSSION

Considerable variations in the total microbial counts in bovine semen have been reported by a number of other workers : in America (Salisbury et al 1939 ; Gunsalus et al 1941 ; Almquist et al 1949a ; Almquist et al 1949b ; Prince et al 1949 ; Foote & Bratton 1950b) , Australia (Singleton & Simmons 1969), Britain (Meredith 1970), Denmark (Hendrikse 1963), India (De & Chowdhury 1965 ; Reddy et al 1971) and Japan (Hashimoto 1963). The microbial populations reported by most of these workers are considerably higher than the populations found in semen from New Zealand bulls (logarithmic average 3.3×10^3 microorganisms/cm^3). This may, in part, be due to the tendency to quote arithmetic rather than logarithmic means, but is probably also associated with the conditions under which the bulls are kept. Intensive housing of the animals leads to greater faecal contamination of the prepuce and hence greater numbers of coliforms present in the voided semen (Singleton & Simmons 1969). In addition, many workers have found that incubation for four days enables a more complete estimation of microbial populations in semen than a two day incubation period. The values obtained during this survey, however, are very close to those obtained during a similar survey in 1972 of artificial breeding herds in New Zealand by Brown et al (1974). In this latter study, all plate counts were incubated for four days, and the microbial populations of semen were found to range from 2×10^1 to 5.34×10^5 microorganisms/cm^3. This compares well with the range reported here of 3×10^1 to 5.6×10^5 microorganisms/cm^3 of semen, using the two day incubation of the total count plates.

The predominant bacterial genera (*Bacillus*, *Corynebacterium* , *Micrococcus*)identified among the selected strains of microorganisms isolated from bovine semen have each been listed as common contaminants of bovine reproductive tract secretions by previous investigators (Gilman 1921 ; Webster 1932 ; Hatziolos 1937 ; Salisbury et al 1939 ; Gunsalus et al 1941 ; Edmondson et al 1948 ; Prince et al 1949 ; Ruebke 1951 ; Sorel 1961 ; Kazda 1963 ; Singleton & Simmons 1969 ; Reddy et al 1971).

The relative frequency with which *Pseudomonas*, *Proteus* and *Escherichia* are detected in semen appears to vary. Gunsalus et al (1941) found *Pseudomonas* and *Escherichia* among the predominant flora ; De & Chowdhury (1965) found coliforms the most common microorganism in bovine semen ; Singleton & Simmons (1969) isolated *Proteus* strains more frequent-

ly than Micrococcus ; and Pseudomonas and Proteus were quite common in bovine semen studied by Reddy et al (1971). Prince et al (1949) found them to be numerically unimportant and in the survey reported here Gram-negative rods appear to be relatively uncommon. The presence of the Staphylococcus sp. is in agreement with Salisbury et al (1939),Gunsalus et al (1941) and Reddy et al (1971), and their low frequency confirms that reported by Singleton & Simmons (1969). High Staphylococcus populations have only been reported by De & Chowdhury (1965). Although streptococci have been reported to be present in bovine semen (Edmondson et al 1948 ; Ruebke 1951 ; Hashimoto 1963 ; De & Chowdhury 1965 ; Singleton & Simmons 1969 ; Meredith 1970) none were identified among the cultures examined in this survey , which is in agreement with Gunsalus et al (1941) and Prince et al (1949). It seems likely that the variation in the genera of microorganisms found in bovine semen under a given set of conditions is dependent on the nature of the microbial population available to invade the genital tract (and also, therefore, on management of the bulls) as well as on the ability of the microorganisms to survive conditions in the preputial cavity and urethra. Those microorganisms occurring with relatively low frequencies cannot be regarded as being true inhabitants of the male reproductive tract. In general they are consistent with the types of micro-organisms which might be present on such materials as bedding, soil, faeces etc., and are probably most accurately described as transient contaminants of bovine semen. This is further supported by the evidence that first ejaculates contain larger microbial populations with a wider variety of colony types than do second ejaculates collected a few minutes after the first. This probably reflects a greater contribution of transient microorganisms washed from the preputial cavity, into the initial ejaculation fluid.

Two of the antibiotics used routinely in semen extenders for artificial breeding techniques, streptomycin and chloramphenicol, were effective against the majority of microorganisms isolated from bovine semen; Penicillin had a low index of effectiveness. It must, however, be recognised that an antibiotic may be important for its ability to inhibit a few specific microorganisms even if its overall effectiveness is low. This is especially significant when considering the efficiency of nystatin against the yeast strains isolates from bovine semen. One of these, Candida guilliermondii, has been implicated in mycotic abortion in cattle (Hugh-Jones & Austwick 1967). Richard et al (1976) also isolated several yeast species from extended semen, the predominant organisms, Candida parapsilosis and Candida tropicalis, have been shown to produce an

endometritis in heifers and, together with Candida krusei, also found in bovine semen, have been the cause of mycotic abortion in cattle. The control of these organisms in semen is therefore of prime importance. In addition the increase in non pathogenic yeast populations seems to determine the usefulness of extended semen stored at ambient temperatures (Brown et al 1974). It is therefore desirable to control fungal as well as bacterial growth in semen used in artificial breeding. Unfortunately nystatin at the concentrations needed to influence fungal growth, depressed conception rates (New Zealand Dairy Board ,1973/1974) and therefore an alternative antifungal agent is required in situations where fungal growth is affecting semen quality.

Among the bacteria isolated from semen the Gram-negative bacteria (Pseudomonas, Proteus, Escherichia) were considerably more resistant to commercial antibiotics than were the remaining bacteria tested. Control of these organisms is most important, since they will grow at 0 to 25'C in extended semen (Foote & Salisbury 1948 ; Foote & Bratton 1950a ; Foote & Bratton 1950b), and have been implicated in bovine reproductive tract disorders (Gunsalus et al 1941 & 1944 ; Prince et al 1949 ; Sorel 1961 ; Zemjanis 1962 ; Kazda 1963 ; Galloway 1964 ; Getty & Ellis 1967 ; Lusis & Soltys 1971). Of the antibiotics normally used in semen extenders, streptomycin appears to be the most effective in inhibiting these bacteria, penicillin has no effect.

Most of the organisms isolated with high frequency from bovine semen are sensitive to the antibiotics used routinely in semen extenders, so that the resistant bacteria are only a relatively small proportion of the population in extended semen. Some strains , however, were found to be resistant to a wide variety of antibiotics,indicating a need for careful management in any treatment given to a bull used for artificial breeding. Practices likely to give such organisms a selective advantage over the normal flora of the urethra and preputial cavity should be avoided. Treatment of an infection in the bulls with antibiotics normally incorporated into semen extenders and the use of preputial washes with these antibiotics should be avoided, so as to prevent the development of a resistant microbial population in ejaculated semen. Such a population would further complicate the problem of controlling microbial growth in semen to be used in artificial breeding services.

The belief that many of the microorganisms present in semen constitute a transient population from outside the reproductive tract is supported by evidence that populations of microorganisms both sensitive

to and resistant to bovine seminal plasma inhibition varied greatly in semen samples collected from the same bull within a few minutes of each other, or at various time intervals, although the measurable antibacterial activity was relatively constant for an individual bull. In addition, the number of organisms resistant to bovine seminal plasma inhibition decreased between first and second ejaculates, whereas sensitive organisms occurred in similar numbers in both samples. The resistant population may reflect the washing out of temporary invaders of the reproductive tract orifice, able to persist there by virtue of their resistance to inhibitors present in tissue fluids and secretions. The second ejaculate might be expected to contain fewer such bacteria, many of which would have been removed in the initial ejaculation fluid. The sensitive population may reflect the opportunities for external contamination of the semen from bedding, soil and faeces by organisms not able to persist for long periods of time in the ejaculated semen or the reproductive tract ; these organisms would constitute a variable proportion of the microbial population in ejaculated semen. A high proportion of the organisms isolated from semen were sensitive to the natural inhibitors present in seminal plasma. Their presence in ejaculated semen probably reflects some measure of protection from the antimicrobial factors present in bovine seminal plasma,by adsorption of active moieties onto other microbial cells, cellular debris and onto sperm cells themselves.

Since the semen of healthy bulls may contain large numbers of microorganisms derived mainly from the lower urethra and preputial cavity, it is likely that potential pathogens may also have access to the male urogenital tract. In spite of this apparent close proximity of potentially infectious microorganisms, the actual incidence of genital infection is low. No bacteria were isolated from the testes, epididymis, ampullae of vas deferens, prostate or bulbourethral glands of healthy animals after slaughter (Ruebke 1951), confirming Gilman's (1921) earlier report that normal healthy animals had reproductive tracts either free of or with very low numbers of microorganisms. Mycoplasmas (Al-Aubaidi et al 1972) and Trichomonas fetus (Parsonson et al 1974b) infections are restricted to the penis and prepuce, rarely ascending the urethra. And the considerable difficulty in isolating both Ureaplasmas (Taylor-Robinson et al 1969) and Infectious Bovine Rhinotracheitis (IBR) virus (Dennett et al 1973) from bovine semen may be associated with inhibitors of these organisms present in the seminal plasma. In the bovine male reproductive tract, testicular abnormalities associated with infection (orchitis, periorchitis and

necrosis), and epididymitis were noted in 1.1 and 1.6% respectively of bulls surveyed by Ladds et al (1973). The tissues of the urogenital tract must therefore, be protected in some way from ascending microbial colonisation.

Antimicrobial compounds occurring naturally in seminal fluid may contribute in such a defence, and protect against deep seated infections of the male urogenital tract. Bovine seminal plasma exhibits antimicrobial activity against a variety of bacteria. Tallgren et al (1968) noted that bovine semen inhibited the growth of Escherichia coli, Proteus mirabilis, Staphylococcus albus[1], and S, aureus, but not Klebsiella sp. or Pseudomonas aeruginosa. Brown et al (1974) reported that Aerobacter, Escherichia, Microbacterium, Micrococcus, Nocardia, Sarcina[2], and some Bacillus and Corynebacterium strains were sensitive to the antimicrobial activity of bovine seminal plasma. Alcaligenes, Proteus, Pseudomonas, Staphylococcus, and some Bacillus and Corynebacterium strains were found to be resistant. In general, these conclusions were confirmed in the present study and in addition strains of : Acetobacter, Arthrobacter, Citrobacter, Enterobacter, Haemophilus, Klebsiella, Leuconostoc, Mycobacterium, Neisseria, Pasteurella, Salmonella, Serratia, Shigella, Streptomyces and Vibrio were inhibited by bovine seminal plasma ; the genera Lactobacillus, Pseudomonas, Staphylococcus and Streptococcus included both sensitive and resistant strains ; the single strains of Nocardia and Providence and all the Proteus strains tested were resistant to bovine seminal plasma.

Discrepancies in the various reports on the effect of bovine seminal plasma on a bacterial species are probably associated with variation in the laboratory strains of the bacterium used for the assay. Thus, the Escherichia coli strain used by Gunsalus et al (1941) and the Staphylococcus aureus strain used by Gurevitch et al (1951), were resistant to bovine seminal plasma, whereas several strains used in this study proved sensitive. The use of such resistant strains as the indicator organism in the earlier experiments led to erroneous reports of a lack of antibacterial activity in bovine semen.

Bacteria resistant to the antimicrobial activity of bovine genital secretions may have a preferential colonising ability for these tissues if the antimicrobial activity serves to prevent urogenital tract infections. Several of the genera in which strains resistant to bovine seminal plasma

FOOTNOTES : [1] currently classified as Staphylococcus aureus (Bergey 1974)
[2] currently classified as Micrococcus (Bergey 1974).

were found, are commonly associated with bovine urogenital tract infections. Pseudomonads have been implicated in seminal vesicular inflammation, epididymitis, orchitis, paraphimosis, phimosis, balanitis and posthetis (Smith et al 1972) and their presence often correlates with sterile semen or semen of low fertility and high sperm abnormalities (Gunsalus et al 1941 ; Gunsalus et al 1944 ; Prince et al 1949 ; Sorel 1961 ; Lusis & Soltys 1971). Staphylococci have been isolated from preputial and scrotal lesions (Mosaheb et al 1973) and cases of seminal vesiculitis (Zemjanis 1962 ; Ladds et al 1973). Sorel (1961) also noted their presence in semen of low fertility and high sperm abnormalities. Streptococci occur in semen from limited fertility bulls (Sorel 1961) and have been isolated from the testes, epididymis and seminal vesicles of bulls with enzootic sterility (Webster 1932). Streptococci are also one of the common causes of infections of the seminal vesicles (Lagerlof 1936 ; Zemjanis 1962 ; Gibbons 1963 ; Galloway 1964) and may give rise to orchitis (Stableforth & Galloway 1959). Providence and Nocardia species are not reported as causes of bovine reproductive tract infections, but Proteus spp. are commonly found in the preputial cavity and may produce opportunistic infections of the higher reproductive tract (Zemjanis 1962 ; Kazda 1963 ; Galloway 1964).

Although, in the present study, the reference strains of Corynebacterium were sensitive to bovine seminal plasma, several strains isolated from semen were resistant to its antimicrobial activity (Brown et al 1974). Bovine epididymitis, orchitis, phimosis, posthetis, preputial /penile and scrotal infections, seminal vesicular lesions and urethritis may be caused by corynebacterial infections (Lagerlof 1936 ; Stableforth & Galloway 1959 ; Donaldson & Aubrey 1960 ; Sorel 1961 ; Zemjanis 1962 ; Gibbons 1963 ; Galloway 1964 ; Smith et al 1972 ; Mosaheb et al 1973 ; Bagshaw & Ladds 1974b). In addition, C. pyogenes often occurs in the semen of bulls with low fertility (Sorel 1961).

Of the bacteria found to be sensitive to bovine seminal plasma, only Escherichia coli has been implicated in bovine urogenital infections. Kazda (1963) and Galloway (1964) noted its presence in inflamed seminal vesicles of bulls with low fertility and seminal vesiculitis.

It seems likely that the natural inhibitors in bovine seminal plasma contribute to the protection of the bovine reproductive tract from infection by many of the microorganisms which invade the preputial cavity. Only those microorganisms capable of growing in the presence of the antimicrobial factors present in bovine genital secretions can successfully

initiate infection of the upper reproductive tract. In this context it is of interest to note that Trichomonas fetus infection is difficult to establish experimentally in bulls, but culture of the organisms in bovine seminal plasma or urine, produces a population better able to infect the bovine reproductive tract (Clark et al 1974b). This enhanced pathogenicity may be the result of selection during growth in the presence of seminal plasma of organisms resistant to the antimicrobial factors associated with the primary non specific defence of the reproductive tract.

In addition, factors other than the pathogen's resistance to a host defence mechanism must be considered when the susceptibility of any individual to bacterial invasion is discussed. Acidic polymers of microbial or tissue origin may neutralise the cationic proteins associated with the primary defence mechanism and thus augment the invasive properties of certain pathogenic organisms. Acidic polymers were found to block the antibacterial activity of cationic proteins from PMN leukocytes (Zeya & Spitznagel 1968). Infection may also result when small defects in the genital secretions such as from trauma, leaves a residue of microorganisms able to invade the urogenital tissues, or from the invasion of micro-organisms in numbers sufficiently large to overwhelm the normal levels of antimicrobial compounds present in the reproductive tract e.g. IBR virus is sensitive to bovine seminal plasma,but can cause venereal disease. Some breakdown in the antimicrobial protection barrier must occur to enable initial colonisation. Once the virus is established in the cells of the reproductive tract they may be protected from the antimicrobial effect of seminal fluid.

Thus, the natural antimicrobial compounds found in bovine reproductive tract secretions, and other tissue fluids,may be important as a primary defence mechanism for the protection of the reproductive tissues from ascending infections of the lower urethra and preputial cavity,where the bacterial populations are substantially higher than elsewhere in the urogenital tract. During coitus they may also serve to protect the female reproductive tract from disease, and possibly to enhance the chances of sperm survival by eliminating some of the competition for nutrients between sperm and the normal flora of the vagina as well as by decreasing the population of microorganisms which are spermicidal (Matthews & Buxton 1951 ; Kazda 1963 ; Lusis & Soltys 1971).

Bacteria sensitive to bovine seminal plasma antimicrobial activity were also rapidly killed by the antimicrobial factor isolated from seminal plasma by adsorption onto DNA. Escherichia coli, Micrococcus lysodeikticus,

and Pseudomonas fluorescens populations were reduced to 0.005 - 0.100% , 33 - 44% and 0.05 - 2.0% of their control populations within 30 minutes respectively.

Direct evidence that bovine seminal plasma antimicrobial activity involves damage to the cell membrane is available from experiments which showed that in the presence of bovine seminal plasma, sensitive cells (B. megaterium and M. lysodeikticus) rapidly lost ^{14}C amino-iso-butyric acid, a non metabolisable analogue of glycine and alanine, did not accumulate valine and lost large amounts of cytoplasmic material. Pseudomonas aeruginosa showed reduced release of cytoplasmic contents,but was able to accumulate valine in the presence of bovine seminal plasma.

The fact that mycoplasmas and certain enveloped viruses were also inactivated by bovine seminal plasma is consistent with the interpretation that bovine seminal plasma antimicrobial activity is associated with the disruption of cell membrane integrity.

No obvious morphological changes occurred in the sensitive bacteria exposed to bovine seminal plasma. The cell wall is not directly affected by bovine antimicrobial activity, its primary effect involves damage to the cell membrane. Shannon et al (1975) reported that when a sensitive strain of Bacillus was exposed to bovine seminal plasma, progressive morphological changes resulted : there was a massive disarrangement of the cell wall material which terminated in the lysis of the cell within a few minutes of contact with the seminal plasma. They concluded that the primary effect of bovine seminal plasma on bacteria involved damage to the cell wall. It is suggested here that this cell wall effect is of a secondary nature, resulting from an autolytis sequel to the initial cell membrane damage in the bacillus studied. Bacillus spp. are known to contain several autolytic enzymes which on release from the cell will produce cell lysis.

Bacterial resistance to bovine seminal plasma is frequently correlated with an increased resistance to polymyxin and colistin sulphate, antibiotics known to interfere with cell membrane integrity. The relatively non specific enhancement of resistance to a variety of antibiotics seen in Gram-negative bacteria may be associated with a general decrease in the permeability of the exterior lipopolysaccharide cell layer in these cells, resulting in the failure of many antibiotics to penetrate the cell and thus reach their site of action. Among Gram-negative bacteria, the cell wall may have some function in protecting the cytoplasmic membrane from bovine seminal plasma activity, since osmotically sensitive forms of both sensitive

and resistant whole bacteria are equally sensitive to lysis by bovine seminal plasma. Treatment with Tris-EDTA and lysozyme destroyed a barrier which normally prevented bovine seminal plasma from reaching the membrane of resistant bacteria. The membrane of Pseudomonas aeruginosa is sensitive to bovine seminal plasma, but the intact cell wall prevents access of the bovine seminal plasma antimicrobial factor to its site of action in undamaged resistant Gram-negative cells. In Gram-positive bacteria, although the cell wall shields the cell membrane,it only impedes the passage of relatively large molecules. In these organisms resistance to bovine seminal plasma may not be associated with a general enhancement of the permeability barrier, but rather with a more specific alteration at the site of action of bovine seminal plasma antimicrobial factor. This type of specific alteration may be reflected in the consistent positive correlation between resistance to bovine seminal plasma and resistance to antibiotics affecting cell membrane integrity among Gram-positive bacteria. Further studies are needed to investigate this possibility.

The information discussed above indicates that bovine seminal plasma acts on susceptible microbial cells by damaging the integrity of their cell membrane and producing massive leakage of cellular contents and ultimate death.

Bovine seminal plasma also appears to increase the fragility of the cell membrane of spermatozoa, resulting in their disintegration and death in certain stress conditions, e.g. centrifugation and rapid cooling (Mann 1964 ; Shannon 1973). Sudden cooling of ejaculated sperm produces rapid irreversible loss of motility, metabolic activity and fertilising function; these effects are associated with permanent damage to the sperm cell membrane, and are similar to those induced by cationic detergents. There is a rapid decrease in the amount of vital metabolites (ATP) present in the spermatozoa, and this is accompanied by a precipitous fall in the rate of fructolysis. Other intracellular protein constituents e.g. cytochrome c , are released more slowly and the lipoprotein complex normally coating the sperm cells becomes detached. Epididymal sperm are more resistant to these effects, but contact with seminal plasma results in a change in antigenic properties associated with the adsorption of proteins onto the sperm cell surface, and renders them susceptible to membrane damage (Mann 1964). The same factor may be responsible for sperm senescence, with increased sperm permeability accompanied by loss of intracellular proteins, K ions and ATP, and swelling and degeneration of the lipoprotein complex normally coating

ejaculated sperm. The agents protecting spermatozoa against the effects of seminal plasma (egg yolk, lecithin), also inhibit the antibacterial effects of bovine seminal plasma (Shannon et al 1975). It is possible that the spermicidal and bacteriocidal effects are both due to the same factor in bovine seminal plasma. Similar factors appear to be present in boar seminal plasma . The seminal vesicles secrete large amounts of cationic proteins (Schellpfeffer & Hunter 1970), and boar spermatozoa are particularly susceptible to cold shock. Davies et al (1975) found that basic proteins secreted by the seminal vesicles adsorbed onto the sperm cells , increased membrane permeability and induced leakage of lactic dehydrogenase and glutamic oxaloacetic transaminase. This membrane alteration rendered the sperm cells very fragile to damage during freezing. Semen from boars minus their seminal vesicles had no difference in their conception rates using unfrozen samples than semen from boars with whole reproductive tract accessory glands. Absence of seminal vesicles did, however, result in an increased conception rate over normal semen when frozen semen was used in artificial breeding. Thus the spermicidal effect of seminal plasma is of little importance under natural breeding conditions and does not affect the efficiency of the normal reproductive process. Sensitivity to cold shock and the detrimental effect of seminal plasma on sperm cells are, therefore, problems associated with artificial breeding techniques ; it is probable that the factors involved in the process are of major importance in disease control of the male reproductive tract, and the prevention of disease transmission during coitus.

The antimicrobial activity of semen has been attributed to a number of normal seminal fluid components, the best known being lysozyme and the polyamines. The polyamines, spermine and spermidine, have been shown to exhibit antibacterial activity (Rozansky, Bachrach & Grossowicz 1954 ; Rozansky, Razin & Grossowicz 1954 ; Grossowicz et al 1955 ; Razin & Rozansky 1959). Spermine occurs in very high concentrations (50 - 350mg/ $100cm^3$) in human semen (Mann 1964) and together with spermidine has been cited as being responsible for some of the antibacterial activity of human semen (Gurevitch et al 1951 ; Razin & Rozansky 1957). Fair & Wehner (1971a) showed that spermine was not effective in inhibiting the growth of bacteria commonly associated with urinary tract infections, and therefore disputed spermine's role in affording protection against bacterial colonisation of the urogenital tract. These organisms are obviously successful in establishing infections in the urogenital tract and can therefore be expected to be resistant to compounds responsible for its defence. This

does not detract from spermine's role in protecting these tissues from a variety of bacteria which might otherwise become established in the reproductive tract. Thus spermine may offer substantial protection for the reproductive tissues from sensitive organisms potentially able to compete successfully with those bacteria known to be responsible for urogenital infections.

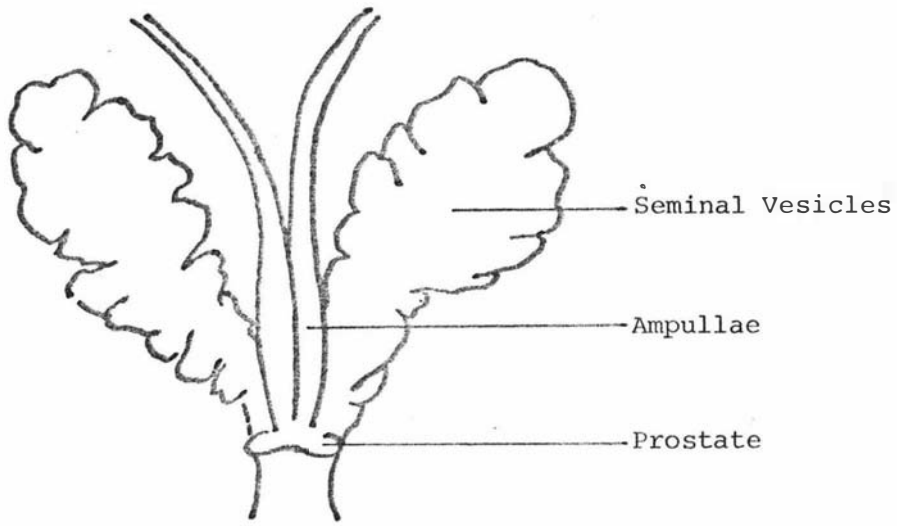
Bovine seminal plasma contains only a trace of spermine, probably due to the absence of true functional prostate glands in the bull (Mann 1964, see FIGURE FORTY TWO). The studies reported here indicate that bovine seminal plasma antimicrobial activity differs substantially from that of spermidine (microbial spectrum, periodate sensitivity etc.). This suggests that spermine and spermidine are not the compounds responsible for the more prominent antimicrobial effects of bovine seminal plasma.

Taylor & Morgan (1952) reported the presence of at least two antimicrobial factors in human semen, spermine and lysozyme or a lysozyme-like compound. Lysozyme derived mainly from the prostate and Cowper's glands, has been reported in human semen (Mardh & Colleen 1974 ; Osserman et al 1974). Many of the reports quoted by Osserman et al (1974) used lysis of Micrococcus lysodeikticus to detect the presence of lysozyme. However, this should not be used as the sole criterion for the identification of an antimicrobial agent as lysozyme, since other compounds also lyse M.lysodeikticus cells. Using an immuno-histochemical technique, Mason & Taylor (1975) detected lysozyme in human kidney tissue, but not in testes or prostate. Thus some of the reported antimicrobial activity of human genital secretions may not be caused by lysozyme.

The presence of lysozyme in bovine secretions has been much disputed. Chandan et al (1968) and Parry et al (1969) detected low levels of lysozyme in bovine milk, but Brownlie & Hibbitt (1972) could not confirm Gibbon's (1959) report of lysozyme in bovine cervical mucus. Its complete absence has also been reported in bovine leucocytes, saliva, nasal exudates and tears (Padgett & Hirsh 1967). This possible absence of lysozyme in bovine seminal plasma together with the fact that in the present study, bovine seminal plasma was found to inhibit mycoplasmas and to be more active against both Gram-positive and Gram-negative bacteria than lysozyme, and the differences in polyacrylamide gel electrophoretic properties between lysozyme and bovine seminal plasma proteins, indicates that lysozyme is not responsible for the antimicrobial activity of bovine seminal plasma.

A bacteriocidal fraction which can be distinguished from lysozyme and spermine has been isolated from canine and human prostatic fluid

BULL :



MAN :

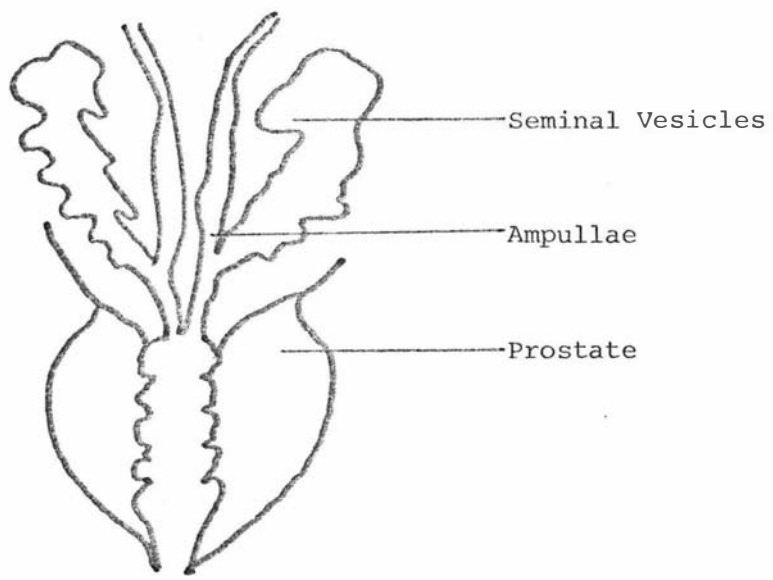


FIGURE FORTY TWO: Accessory Glands of the Male Reproductive Tract of Bull and Man (from Mann 1964).

(Stamey et al 1968). It was characterised as a heat stable, trypsin insensitive compound of low molecular weight. This is possibly the same compound described by Fair & Wehner (1971b), when they eliminated spermine as being the active antibacterial agent in canine prostatic fluid. Youmans et al (1938) described another antibacterial factor in canine prostatic fluid, which was completely destroyed at 60'C for 30 minutes. Bovine seminal plasma antimicrobial activity is heat stable at 60'C and is sensitive to trypsin. Consequently the inhibitors present in bovine seminal plasma also appear to be distinct from those found in the seminal plasma of dogs and man. Further characterisation of these compounds would, however, be desirable, allowing further comparison between compounds isolated from the various genital secretions.

It is not surprising, however, that different compounds present in the genital secretions of different species may perform similar biological functions. There are profound differences in the size and morphology of homologous accessory glands in various species, and wide species variations therefore exist in the volume and composition of individual secretions and the relative proportions of the genital secretions originating from the various secretory organs. Because substantial amounts of certain compounds are restricted to the seminal plasma of only a few species, it does not detract from the physiological importance ; other compounds may perform similar functions in another species. For example, in human semen the citrate and calcium concentrations are greater than, and the chloride content less than those present in canine semen. However, the total concentration of osmotically active substances are of the same order in the secretions of both animals. Similarly, the primary defence mechanism for the male reproductive tract in different species may reflect the metabolic capabilities of the animals concerned ; lysozyme and polyamines may well be responsible for the antimicrobial activity of human semen, while other unique cationic proteins may play a similar role in bovine semen.

Shannon et al (1974 & 1975) have studied bovine seminal plasma antibacterial activity and described a heterogeneous complex, involving a small molecular weight polypeptide monomer and various molecular weight polymers with antibacterial activity. The actual amount of monomer was small in relation to the total antibacterial activity. Similar compounds were isolated from bovine spleen, pancreas, liver and lung tissue.

In this study, evidence has been obtained for the presence in bovine seminal plasma of several different compounds with antimicrobial activity.

The antiviral activity responsible for the neutralisation of Herpes and infectious bovine rhinotracheitis (IBR) viruses is heat labile and possibly associated with the toxicity of bovine seminal plasma for cell cultures. This toxicity was also reported by Spradbrow (1968). The antiviral activity appears identical with that discussed by Le Q. Darcel & Coulter (1976). They confirmed Bitsch's (1964) report that bovine seminal plasma exhibits activity against IBR virus. Both the virus neutralising activity and the toxicity to tissue culture were destroyed by heating bovine seminal plasma to 56'C for 30 minutes, and were readily adsorbed onto kaolin. It therefore appears that the antiviral and antibacterial activity activity in bovine seminal plasma are associated with different compounds.

An extremely heat labile compound or complex also occurs in bovine seminal plasma and is associated with a seminal plasma inhibitory effect on some Staphylococcus aureus strains. The level of this activity in bovine seminal plasma varies with individual samples. Bacillus subtilis is also inhibited by a heat labile compound in bovine seminal plasma, while bovine seminal plasma proteins have a bacteriostatic rather than bacteriocidal effect on this organism. Further characterisation of this compound was not attempted.

The activity which was investigated further in this thesis is heat stable at 60'C for 60 minutes and has a lethal effect on both Gram-positive and Gram-negative bacteria as well as on mycoplasmas. It is probably identical with the heat stable nondialysable mycoplasmicidal compound found in bovine serum and semen by Taylor-Robinson et al (1969). The partial loss of this activity against Micrococcus lysodeikticus which occurred at higher temperatures was probably due to non specific precipitation of the active fraction with other heat labile seminal proteins, or to a certain fraction of the antimicrobial compound occurring in a heat labile association.

The decrease in the antibacterial activity of bovine seminal plasma after treatment with proteolytic enzymes may be due in part to the release of compounds from the proteolysis of other bovine seminal plasma constituents which act as inhibitors of the antibacterial activity. There is no doubt, however, that bovine seminal plasma antimicrobial activity is subject to degradation by several proteolytic enzymes and is a protein molecule itself. This activity is unaffected by periodate treatment, indicating that carbohydrate moieties are not essential for its function as an antimicrobial agent.

Dialysis and Ultrafiltration of bovine seminal plasma suggest that the antimicrobial factor exists in more than one form (as reported by Shannon et al (1974 & 1975) in their studies on bovine seminal plasma antibacterial activity). Dialysable molecules with antibacterial activity exist in equilibrium with larger non dialysable complexes which can dissociate to release the smaller dialysable moiety. This smaller moiety is able to reaggregate into complexes of molecular weights greater than 50,000 daltons, since a portion of the antibacterial activity having passed through an ultrafiltration membrane failed to do so on re-filtration. The non dialysable nature of the mycoplasmicidal activity described by Taylor-Robinson et al (1969) may be associated with the larger molecular weight aggregates of the active factor commonly occurring in bovine seminal plasma.

The measurable amounts of antibacterial activity present in bovine seminal plasma may to some extent be associated with the nature of the active fraction present in whole seminal plasma. Some of the activity is probably adsorbed onto sperm and bacterial cells, and other seminal proteins. The extent of this adsorption and its reversibility may affect the biological assay of the antibacterial activity present in a given sample. Thus, it seemed best to regard the levels recorded in the assay system as a measure of functional activity rather than the absolute concentration of such activity in bovine semen or seminal plasma. The level of antibacterial activity seen in semen and seminal plasma samples may therefore reflect not only the intrinsic ability of a bull to produce the compounds involved but also the proportion of activity available for active participation in microbial inhibition.

In the agar well diffusion test there is the additional provision that the assay is dependent on the ability of the active moiety to diffuse through the agar, and hence the state of aggregation of the active factor could affect the assay results. The increase in the diameter of inhibition zones produced by bovine seminal plasma and semen samples in the presence of citrate may be due to (a) increasing the ability of the antimicrobial factor to diffuse through the agar due to neutralisation of charges on the agar or by altering the charge carried by the molecule itself. From the behaviour in native polyacrylamide gel electrophoresis it is apparent that citrate modifies the migration of the antimicrobial factor in an electric field ; the electrophoretic properties are consistent with a conversion from a cationic to an anionic state.

(b) increasing the dissociation by citrate

of higher molecular weight aggregates present in bovine seminal plasma to smaller moieties would also increase the ability to diffuse through the assay agar.

(c) citrate may affect the specific activity of the factor by increasing the sensitivity of the test organism to the antimicrobial factor. Such an effect might be tested in an alternative liquid assay system, where the ability of the factor to diffuse is not significant to the assay.

Sephadex SP_C25 treatment of bovine seminal plasma proteins substantially increased the diameter of the inhibition zone produced. This could be due to the removal of inhibitory compounds normally bound to a certain fraction of the antimicrobial proteins in bovine genital secretions, or to removal of a factor which favours the association of active moieties into aggregates whose activity is not measured in the assay system being used. Native polyacrylamide gel electrophoresis of the treated material produced a streak of cationic material, this may indicate alterations in the aggregate forms of the active moiety, possibly by removal of an inhibitory compound from the complex.

The variation in size of the protein peaks eluted from Amberlite IRC-50 cationic exchange resin by Tris or phosphate buffers is thought to represent changes in the physical state of the molecule responsible for the antimicrobial activity ; dialysis of highly purified material against phosphate buffer also produced alterations in the electrophoretic migration of the isolated protein (CHAPTER 20.8) . The differential release of active fractions from the Amberlite IRC-50 resin probably represents different molecular weight complexes of the active moiety having varying affinities for the cationic exchange resin. With the present understanding of the properties of the protein responsible for some of the antimicrobial activity in bovine seminal plasma, it may be possible to use an ion exchange resin in a procedure similar to that developed with DNA (CHAPTER 19.4).

Native polyacrylamide gel electrophoresis showed no resemblance between the migratory patterns of bovine seminal plasma proteins, and those of lysozyme, polylysine, salmine or spermidine. Bovine seminal plasma produced several cationic protein bands at pH 8.6, which exhibited anti-bacterial activity by the overlay technique used to locate biological activity in single bands of electrophoresed proteins. These active proteins separable by electrophoresis are probably aggregates produced by self -

association of the active moiety into several different molecular weight complexes.

The antimicrobial factor effective against both Gram-positive and Gram-negative bacteria and mycoplasmas was isolated from bovine seminal plasma by adsorption of the active factor onto highly polymerised DNA. Some activity was not adsorbed during the extraction process, and this might represent another heat stable factor present in bovine seminal plasma, or certain aggregates of the isolated material having little affinity for DNA. There is indeed some indication that the physical state of the purified antimicrobial factor may vary with the ionic environment which is present, existing in at least two molecular weight forms in equilibrium with one another.

The protein isolated from bovine seminal plasma by adsorption onto DNA is trypsin sensitive (although not as sensitive as whole bovine seminal plasma,possibly due to the non specific inhibition of the active moiety by endproducts of trypsin digestion of other seminal plasma proteins). It is heat stable (60'C for 60 minutes) and has no carbohydrate moiety associated with its active site.

In Ultracentrifugation and native polyacrylamide gel electrophoresis studies it was found that there is considerable variability in the physical state of the protein, due to both self-association and to interactions with the ionic microsolutes present in its environment. Thus phosphate dialysis produced a single protein species on native polyacrylamide gel electrophoresis, which after several weeks reassociated to produce four bands on electrophoresis at pH 8.6 . These migratory properties probably reflect interactions between the active protein moiety and the multivalent phosphate ions in the buffer solution, producing a variety of differing complexes. Similar associations and dissociations can be induced by heat, low pH, the addition of citrate etc. In bovine seminal plasma it is very likely that the active protein is also found in associations with other seminal plasma constituents : proteins, lipids and carbohydrates.

On N terminal amino acid analysis the purified material was found to possess a single N terminal residue, glutamic acid or its amide derivative. It also migrated as a single protein band under SDS polyacrylamide gel electrophoresis, which suggests the presence of a single protein moiety, migrating at a rate consistent with a molecular weight of approximately 25,000 daltons. This antimicrobial factor has a high content of aromatic amino acids (phenylalanine, tyrosine) and histidine, but no

arginine residues in its composition. Its basic nature must therefore be a function of its high lysine and histidine residues together with amide derivatives of the acidic amino acids present.

There have been several reports in the literature of cationic proteins possessing antimicrobial properties and possibly acting as primary non specific defence mechanisms in various regions of animal anatomy,and of antimicrobial activities of various systems possibly associated with the secretion of such components, although a complete characterisation of the agents involved has not been made.

A powerful antibody independent, antivibrio mechanism exists at the epithelial surface of mouse intestine, not associated with the indigenous flora of the region and requiring actively metabolising cells. It is thought to be associated with the secretion of some component which has a bacteriocidal effect on Vibrio cholerae (Knop & Rowley 1975).

The bladder antibacterial defence system is also associated with the secretion of compounds which prevent the initial binding of bacteria to the bladder mucosa and kill those organisms in direct contact with the mucosa (Lowell Parsons et al 1975).

A heat stable (60'C) fraction active against several mycoplasma (including ureaplasma) species has been found to occur in normal bovine whey (Howard et al 1975), and in bovine serum and semen (Taylor-Robinson et al 1969). Chemical characterisation was not attempted.

Antimicrobial proteins have been reported in numerous tissues and fluids, but most differ from the activity isolated from bovine seminal plasma in heat stability, activity spectrum or mode of action. The seminal plasma activity is heat stable at 60'C and is therefore not associated with complement (Muller-Eberhard et al 1966).

Histones and protamines (Hirsch 1958 ; Busch 1965 ; Phillips 1971) are basic proteins with antimicrobial activity, producing no detectable lysis or morphological alterations of the bacterial cell. They readily form aggregates of high molecular weights, with sedimentation coefficients of 80 - 280 S between pH 5.6 and pH 8.1 . In these characteristics they resemble the material present in bovine seminal plasma, but several differences do exist (TABLE XXXII). In addition, their antibacterial activity is decreased by the presence of serum or albumin, changes in pH (no activity at pH 7.0) and increasing ionic strength decreases the

TABLE XXXII.

COMPARISON OF THE PROPERTIES OF HISTONES WITH THOSE OF THE ANTIMICROBIAL FACTOR ISOLATED FROM BOVINE SEMINAL PLASMA.

PROPERTY	HISTONE	BOVINE SEMINAL PLASMA FACTOR
N terminal amino acid *	alanine, proline	glutamic acid
Basic amino acids *	more than 22%	11%
arginine *	high	absent
cysteine *	low	intermediate
tyrosine *	absent	high
Charge at pH 8.0	positive	positive
Kill in 0.15M NaCl *	no activity	high activity
Kill with serum present *	decreased	no effect
Response of		
Escherichia coli	sensitive	sensitive
Klebsiella pneumoniae	sensitive	sensitive
Proteus morganii	resistant	resistant
Pseudomonas aeruginosa *	sensitive	resistant
Serratia marcescens *	resistant	sensitive
Staphylococcus aureus *	sensitive	variable
Streptococcus haemolyticus *	resistant	sensitive

FOOTNOTE : * properties which differ between bovine seminal plasma antimicrobial factor and histones.

killing capacity of histones (no kill occurs at 0.15M NaCl). This differs considerably from the active material isolated from bovine seminal plasma.

Interferon may in fact be a histone (Harold 1970), but it inhibits viral replication by altering the host cell metabolism rather than having a direct effect on the virus or viral activity. It is not known to be anti-bacterial.

Lactenin, found in bovine, caprine and human whey, has specific activity against group A Streptococci (Wilson & Rosenblum 1952).

Lactoferrin and transferrin act by preventing bacterial growth in the host by a mechanism interfering with normal bacterial iron metabolism i.e. act by nutritional immunity. It is heat stable (56'C), bacteriostatic and pH dependent (no activity at pH 6.8) (Oram & Reiter 1968 ; Bullen et al 1972).

Lysozyme does not appear to be important as a primary defence mechanism of cattle (see discussion above).

Polymyxins are heptapeptides with a long carbohydrate side-chain (Harold 1970) which are more effective against Gram-negative than Gram-positive bacteria ; they bind to phospholipids of the lipopolysaccharide layer and plasma membrane, to produce a re-orientation of the membrane lipids and a breakdown of the permeability barrier.

Properdin is bacteriocidal and antiviral (Wardlaw & Pillemer 1956), but is heat labile (56'C) and requires Magnesium ions and complement.

Tyrocidines are cyclic peptides, strongly basic, with the basic residues essential for antimicrobial activity and hydrophobic residues needed to bind the molecule to the membrane. They do not solubilise the whole bacterial membrane, but disrupt the permeability barrier and cause the release of small metabolites from affected cells (Harold 1970).

The smallest peptide fragment with antimicrobial activity appears to be tripeptides with the general formula L arginine - D x - L phenylalanine, which have been found to have antifungal activity (Eisele 1975). Such a sequence may be similar to those present in the active sites of other larger basic peptides and proteins.

Groups of cationic proteins have been isolated from many biological systems, and have been associated with primary defence mechanisms in several animal species.

The primary function of neutrophilic granulocytes is phagocytosis and destruction of invading microorganisms. A group of heat stable (100'C for 10 minutes) cationic proteins have been isolated from human neutrophilic granulocytes with antibacterial (Odeberg et al 1975) and antifungal (Lehrer et al 1973) activity. Olsson & Venge (1974) differentiated these proteins into two separable groups. Group one (components 1 - 4) had molecular weights of 25,500 to 28,500 daltons, almost identical amino acid compositions, and immunochemical identity also. The other group (components 5 - 7) had molecular weights of 21,000 to 29,000 daltons, and also showed immunochemical identity. Lehrer (1973) distinguished several components from human neutrophils with selective antibacterial effects. Five constituents were active against Staphylococcus aureus, three against Bacillus subtilis, two against Escherichia coli and one active against Klebsiella pneumoniae. The fractions active against S. aureus had no effect on Gram-negative bacteria. These cationic proteins from human neutrophilic granulocytes had higher molecular weights and lower basic amino acid compositions than similar proteins isolated from PMN leukocytes from other species.

Lysozyme and other cationic proteins are associated with the antibacterial activity of peroxidaseless chicken polymorphonuclear (PMN) leukocytes (Brune & Spitznagel 1973). In guinea pig PMN leukocytes,the cationic proteins have approximately 25% basic residues in their overall amino acid composition, and markedly different compositions from those of nuclear histones (Zeya & Spitznagel 1966b) ; they are effective against both Gram-positive and Gram-negative bacteria including Bacillus megaterium, Candida albicans, Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus, and they interact with and disorganise bacterial structures responsible for the maintenance of the normal permeability barrier (Zeya & Spitznagel 1966a). On resolution the cationic proteins from rabbit PMN leukocytes exhibit preferential inhibitory activity against different bacterial species, as well as chemical heterogeneity (Zeya & Sptiznagel 1968). These proteins are low in aromatics, have high arginine : lysine ratios, and a high cysteine content. Their activity may be blocked by the presence of iron and haematin but not haemoglobin (Gladstone & Walton 1971).

Cationic proteins are also associated with the myeloperoxidase - independent candidacidal mechanisms in human monocytes, active against Candida pseudotropicalis and Candida parapsilosis, but not against Candida albicans (Lehrer 1975).

Antimicrobial, heat stable (70'C) basic proteins have been isolated from bovine neutrophils (Brownlie 1971), and from the cells (epithelial,

neutrophilic, basophilic, lymphocytic and monocytic) of bulk bovine milk samples (Hibbitt et al 1971). These may be released from the cells into the milk and constitute the initial defence of the mammary gland against invading microorganisms.

The teat canal keratin of the cow also contains cationic proteins with antimicrobial activity (Hibbitt & Cole 1968 ; Hibbitt et al 1969). These have been shown in in vitro studies to inhibit the growth of mastitis strains of staphylococci and streptococci. The proteins adsorb onto the cell surface by electrostatic interaction (Hibbitt et al 1969 ; MacMillan & Hibbitt 1973) and are bound to the cell wall and membrane with little protein penetrating into the cytoplasm (MacMillan & Hibbitt 1973). This adsorption results in a thinning or complete disruption of the cell wall, disorganisation of the cell membrane, accumulation of fibrillar material on the outer surface of the bacteria, and some degree of plasmolysis (MacMillan & Hibbitt 1969). This disruption of components responsible for the osmotic barrier of the cell results in the rapid leakage of isotopically labelled protoplasmic proteins from bacterial cells (Hibbitt & Benians 1971 ; Hibbitt et al 1972). In vivo, similar adsorption, fine structural changes and eventual kill occur in staphylococci collected from the teat canals of cows after close proximity to the keratinised epithelial cells for 24 hours (MacMillan & Hibbitt 1969 ; Hibbitt & Benians 1971). Thus the teat canal of healthy cows provides an environment continually repressive to microbial growth and the cationic proteins produced by this tissue provide the initial line of natural defence for the mammary gland, as well as rendering the invading microorganisms more susceptible to the activity of phagocytic polymorphs and immune globulins.

Non serum antimicrobial cationic proteins have also been isolated from bovine cervical mucus and are thought to be responsible for the local non specific resistance of the female reproductive tract to bacterial invasion. For example, the cervix appears to be a barrier against the spread of brucellosis from infected bulls to susceptible cows by natural infection, although artificial intrauterine insemination with Brucella contaminated semen will transmit brucellosis (Brownlie & Hibbitt 1972).

The antimicrobial protein isolated from bovine seminal plasma by adsorption onto DNA, appears to be of a unique chemical composition, different from the amino acid profiles of other cationic proteins which have been characterised (Zeya & Spitznagel 1966b ; Zeya & Spitznagel 1968), see TABLE XXXIII . It differs from nuclear histones, polymorphonuclear cationic proteins and separated peak 4 PMN cationic proteins in the absence

TABLE XXXIII.

COMPARISON OF THE PERCENT AMINO ACID COMPOSITION OF CATIONIC PROTEINS FROM POLYMORPHONUCLEAR LEUKOCYTES, HISTONES, AND THE ANTIMICROBIAL FACTOR ISOLATED FROM BOVINE SEMINAL PLASMA.

AMINO ACID	DNA ISOLATE	PMN CATIONIC PROTEINS*	TOTAL NUCLEAR HISTONES*	PEAK 4 OF PMN CATIONIC PROTEINS #	
				anti-staphylococcal	anti-streptococcal
aspartic acid	12.7	7.6	5.6	1.7	0.4
glutamic acid	9.5	9.2	8.3	3.9	4.0
glycine	8.2	6.2	8.6	6.2	5.6
alanine	7.0	6.5	12.9	9.4	8.4
valine	4.4	5.9	6.5	4.0	4.2
leucine	7.0	9.5	8.1	9.8	10.1
isoleucine	2.5	4.9	4.2	6.1	7.0
serine	7.6	3.4	4.1	1.7	1.4
threonine	5.7	5.7	5.5	0.8	0.8
cysteine	5.7	3.5	0.4	13.5	14.0
methionine	1.3	1.1	1.1	0.4	0.2
proline	4.4	6.3	5.1	7.0	6.6
phenylalanine	6.3	4.2	1.8	2.9	2.3
tyrosine	6.3	1.2	2.4	0.7	0.0
histidine	1.9	0.7	2.8	2.7	3.4
lysine	9.5	8.6	13.8	3.8	0.9
arginine	0.0	15.6	8.9	25.4	30.8

FOOTNOTES : * data from Zeya & Spitznagel (1966b)

data from Zeya & Spitznagel (1968).

of arginine, and in the higher numbers of serine and aspartic acid residues. The bovine protein tends to have a lower proline content than the other cationic proteins, but phenylalanine and tyrosine are substantially higher ; the content of glutamic acid, methionine and threonine is similar to the proteins from PMN leukocytes and nuclear histones, but greater than that of peak 4 isolated proteins from PMN leukocytes. The level of cysteine in the protein of bovine origin is higher than that for the PMN cationic proteins, but rather lower than that for separated peak 4 proteins from this group. Histones contain the largest amounts of lysine, bovine seminal plasma and PMN cationic proteins have intermediate lysine contents and the isolated peak 4 proteins from PMN leukocytes contain the least.

Thus, proteins associated with non specific antimicrobial resistance in several animal species, although chemically heterogeneous, are able to perform similar biological functions, producing disturbances of the osmotic state of the cell and inducing massive leakage of cellular contents. Variation in their exact chemical composition may be associated with antibacterial specificity. These proteins, which are cationic at physiological pHs, are important in the initial non specific defence against microbial invasion via the natural orifices of the animal body, and have been found in the biological components of several animal systems. Other constituents of these systems, especially if of an anionic nature, may result in apparent inhibition of the active cationic proteins e.g. whole keratin had no antibacterial activity, since the active moiety was bound to other negatively charged compounds which were present (Hibbitt et al 1969). However, some of the complexes of the antimicrobial factor with other components, for example of seminal plasma, or self-aggregations with other monomeric forms of the factor, may act as a stable reservoir from which the small active moiety can be withdrawn as required by the dissociation of these complexes. Such associations may also serve to render the active protein less susceptible to metabolism or elimination from the body. In addition, within the living animal cationic proteins are being systhesised continually and are therefore always available to bind to anionic material including invading microorganisms.

Skarnes & Watson (1957) believed that antimicrobial cationic proteins were only present in normal tissues in response to the physiological changes accompanying stress. There is now overwhelming evidence that such compounds are present in many normal tissues and secretions in the absence of stress conditions. Although evidence is accumulating that under the

conditions of specific challenge the amounts produced by normal tissues may increase substantially. Thus , Brownlie (1971) noted an increase in antimicrobial cationic proteins both in bovine neutrophilic granulocytes and free in the milk from the mammary glands of animals with induced sterile mastitis. Endotoxin injection into mice resulted in an enhanced non specific resistance to bacterial infections probably associated with the release of cationic proteins into the intercellular fluids (Hill et al 1973). In addition, the inflammatory response of rabbits to endotoxin injection has been shown to stimulate the accumulation of large amounts of bacteriocidal compounds (beta-lysin and lysozyme), released from platelets and leukocytes into tissues where measurable amounts of these compounds are not normally present (Tew et al 1971).

Bovine seminal plasma antimicrobial cationic proteins appear to be of a unique character compared with other similar compounds which have been investigated, although they may reasonably be assumed to perform similar biological functions, i.e. they act as primary natural non specific defence mechanisms in those areas of the body readily exposed to microbial invasion.

These proteins provide an effective defence of the bovine male reproductive tract since few microorganisms are isolated from the upper genital tract of healthy animals ; most microorganisms are restricted to a colonisation of the penis and prepuce below the accessory glands which are thought to produce the antimicrobial secretions. In addition, ascending infection from the large reservoir of potential pathogens in the preputial cavity is rare, and reproductive disorders are uncommon. The causitive agents are usually those microorganisms which are resistant to the antimicrobial activity of bovine seminal plasma. The considerable difficulty experienced in isolating Ureaplasmas (Taylor-Robinson et al 1969) and IBR virus (Parsonson et al 1974b) from bovine semen, and the enhancement of Trichomonas fetus infectivity by culture in bovine seminal plasma, may also reflect the ability of the male reporductive tract and its secretions to remain relatively sterile. The antimicrobial compounds also repress the transmission of venereal diseases to serviced cows, decrease the spermicidal microbial population in semen and restrict the number of bacteria potentially able to cause endometritis and foetal abortion.

Similar cationic proteins have also been found in bovine spleen, pancreas, liver and lung tissue (Shannon et al 1975) suggesting that these compounds are part of the overall non specific defence mechanisms

throughout the bovine body, protecting the natural orifices from invasion by potentially pathogenic microorganisms.

FUTURE RESEARCH :

I would like to consider that future studies of the antimicrobial compounds in bovine seminal plasma will proceed along several lines of investigation.

For the artificial breeding industry, emphasis would be on the identification of the precise components functioning as sperm toxic agents, and the study of blocking agents which would enable the long term storage of semen without present difficulties associated with cold shock of sperm. This is especially true if the information gained in the bovine system is applicable to boar and ram artificial breeding programmes, where successful artificial breeding techniques have still to be fully developed.

A more general study of primary non specific defence mechanisms requires the identification of the other antimicrobial components of bovine seminal plasma, including the antiviral compound and the heat labile anti-staphylococcal activity (this may be associated with antibody-complement interactions).

Closer studies of the isolated factor, mode of action in relation to its active site, the mechanisms of bacterial resistance etc., could lead to a long term assessment of its potential as a commercial antibiotic, depending on the reactivity of the host as well as on the effectiveness of the compound as an antimicrobial agent.

Physiological studies must be attempted to locate the site of production in the reproductive tract, the precise cells involved in its secretion and factors influencing its production. In a given individual, marked fluctuations in quality, quantity and composition of secretions of the accessory sex glands may reflect the functional activity of that particular gland, governed primarily by hormones of testicular origin. The output of androgens is subject to control by the anterior hypophysis and hence many factors (age, light, season, temperature, diet) may ultimately affect the antimicrobial activity of an animal's genital secretions. Corticosteroids (dexamethasone) enhanced the ability to isolate IBR virus from bovine semen (Clark et al 1974b). The precise nature of the effect of this synthetic hormone on genital secretion and IBR virus in not known, but indicates the need to investigate the effect of hormonal activity on bovine genital tract antimicrobial secretions.

In other systems where cationic proteins have antimicrobial functions, there is some indication that challenge by endotoxin, and possibly invading microorganisms stimulated the production of and release of cationic

proteins. Such investigations of the bovine reproductive tract may provide additional information on the response of non specific natural antimicrobial systems to disease.

APPENDIX ONE

MICROBIAL NUMBERS AND ANTIBACTERIAL ACTIVITY IN INDIVIDUAL BULL SEMEN SAMPLES.

BULL	DATE	ANTIBACTERIAL ACTIVITY		MICROBIAL POPULATION			
		Inhibition Zone (mm)	LzU_e/cm^3	Total Count	(a)[1]	(b)[2]	(c)[3]
Fresian:							
940	31/10	19.75	18,250	45,000	-	-	-
	6/11	19.75	18,250	550,000	-	-	-
	11/11	21.00	27,250	160,000	159,000	1,000	5
5211	31/10	20.00	20,000	2,400	-	-	-
6429	14/11	21.50	31,000	270	110	160	3
6463	13/11	20.50	23,250	290	260	30	2
7200	31/10	18.75	13,250	6,500	-	-	-
	3/11	20.75	25,000	7,800	-	-	-
	6/11	19.50	17,000	38,000	-	-	-
7205	5/11	21.50	31,000	1,200	-	-	-
	8/11	19.50	17,000	1,800	-	-	-
7221	2/11	18.50	12,250	300	-	-	-
	5/11	20.00	20,000	440	-	-	-
	9/11	16.50	6,400	6,000	5,100	900	2
	15/11	19.00	14,250	31,000	30,900	100	3
7443	12/11	21.75	33,500	2,700	2,600	100	6
9230	1/11	19.25	15,750	240	-	-	-
	4/11	19.00	14,250	2,200	-	-	-
	7/11	15.75	4,800	4,600	-	-	-
	13/11	19.25	15,750	3,900	3,400	500	7
	16/11	19.25	15,750	7,300	6,200	1,100	3
	19/11	19.75	18,250	3,500	3,400	100	6
	22/11	18.25	11,250	240,000	240,000	0	3
9489	12/11	20.00	20,000	20,000	20,000	0	3

/ . . .

APPENDIX ONE (contd)

BULL	DATE	ANTIBACTERIAL ACTIVITY		MICROBIAL POPULATION			
		Inhibition Zone (mm)	LzU_e/cm^3	Total Count	(a)[1]	(b)[2]	(c)[3]
Hereford:							
72610	12/11	19.25	15,750	800	750	50	5
	18/11	18.00	10,500	41,000	41,000	0	1
72611	2/11	15.25	4,000	6,300	-	-	-
	7/11	18.25	11,250	490	-	-	-
	10/11	16.00	5,350	2,000	0	2,000	1
	16/11	18.25	11,250	2,600	2,400	200	4
72613	3/11	18.00	10,500	50	-	-	-
	6/11	18.00	10,500	700	-	-	-
	9/11	17.00	7,500	1,600	1,590	10	3
	15/11	16.50	6,400	7,100	200	6,900	2
	21/11	18.75	13,250	88,000	88,000	0	2
72624	5/11	17.25	8,200	700	-	-	-
	8/11	16.50	6,400	260,000	-	-	-
	14/11	17.00	7,500	200	38	162	5
	17/11	18.00	10,500	4,200	4,100	100	2
	20/11	17.75	9,600	30	30	0	1
Charolais:							
66076	1/11	20.00	20,000	17,000	-	-	-
	3/11	19.25	15,750	750	-	-	-
	5/11	20.50	23,250	6,000	-	-	-
	7/11	20.25	21,500	26,000	-	-	-
	9/11	17.50	9,000	10,000	6,900	3,100	4
	11/11	21.50	31,000	830	520	310	3
	13/11	19.50	17,000	560,000	560,000	0	5
	15/11	20.50	23,250	33,000	19,000	14,000	6
	17/11	19.75	18,250	1,400	790	610	2
	19/11	21.75	33,500	7,400	7,400	0	4
	21/11	19.50	17,000	150,000	130,000	20,000	8

/ . . .

APPENDIX ONE (contd)

BULL	DATE	ANTIBACTERIAL ACTIVITY		MICROBIAL POPULATIONS			
		Inhibition Zone (mm)	LzU_e/cm^3	Total Count	(a)[1]	(b)[2]	(c)[3]
66175	2/11	16.00	5,350	18,000	-	-	-
	4/11	19.00	14,250	9,200	-	-	-
	6/11	20,00	20,000	2,900	-	-	-
	8/11	19.25	15,750	170	-	-	-
	10/11	20.25	21,500	230	160	70	3
	12/11	20.75	25,000	650	200	450	6
	14/11	21.50	31,000	1,000	580	420	3
	18/11	22.00	36,000	460	360	100	9
	20/11	19.50	17,000	16,000	30	15,970	3
	22/11	20.25	21,500	1,100	20	1,080	4

FOOTNOTES: [1] (a) = number of organisms resistant to the natural antibacterial activity of bovine semen.

[2] (b) = number of organisms sensitive to the natural antibacterial activity of bovine semen.

[3] (c) = number of different microbial colony types in each sample.

APPENDIX TWO

MICROBIAL NUMBERS AND ANTIBACTERIAL ACTIVITY IN PAIRED EJACULATES FROM INDIVIDUAL BULLS.

BULL	DATE	EJACULATE NUMBER	ANTIBACTERIAL ACTIVITY		MICROBIAL POPULATION			
			Inhibition Zone (mm)	LzU_e/cm^3	Total Count	(a)[1]	(b)[2]	(c)[3]
5211	3/11	first	19.50	17,000	1,600	-	-	-
		second	20.00	20,000	1,100	-	-	-
	6/11	first	19.50	17,000	3,300	-	-	-
		second	18.25	11,250	3,900	-	-	-
	9/11	first	17.75	9,600	4,700	3,200	1,500	5
		second	17.75	9,600	5,600	2,900	2,700	3
	12/11	first	20.00	20,000	5,100	3,000	2,100	4
		second	20.50	23,250	5,000	0	5,000	4
	15/11	first	19.00	14,250	3,100	2,400	700	4
		second	20.00	20,000	500	400	100	3
	18/11	first	20.75	25,000	50,000	49,000	1,000	5
		second	20.50	23,250	3,900	1,700	2,200	4
	21/11	first	20.00	20,000	2,700	2,670	30	7
		second	21.50	31,000	2,500	2,200	300	3
6200	1/11	first	17.25	8,200	16,000	-	-	-
		second	18.50	12,250	4,000	-	-	-
	4/11	first	18.25	11,250	12,000	-	-	-
		second	19.25	15,750	3,000	-	-	-
	7/11	first	18.75	13,250	5,100	-	-	-
		second	20.00	20,000	2,000	-	-	-
	10/11	first	19.50	17,000	6,700	6,600	100	4
		second	19.50	17,000	5,500	4,700	800	2

/ . . .

APPENDIX TWO (contd)

BULL	DATE	EJACULATE NUMBER	ANTIBACTERIAL ACTIVITY		MICROBIAL POPULATION			
			Inhibition Zone (mm)	LzU_e/cm^3	Total Count	(a)[1]	(b)[2]	(c)[3]
6200 . . .	13/11	first	18.75	13,250	1,400	650	750	5
		second	19.25	15,750	2,100	1,900	200	3
	16/11	first	19.75	18,250	79,000	72,000	7,000	4
		second	20.50	23,250	4,900	4,600	300	3
	19/11	first	19.00	14,250	2,000	1,900	100	3
		second	20.50	23,250	90	90	0	3
	22/11	first	20.50	23,250	100	50	50	2
		second	19.50	17,000	35,000	16,000	19,000	4
7200	8/11	first	18.25	11,250	17,000	-	-	-
		second	18.50	12,250	1,600	-	-	-
	14/11	first	20.75	25,000	85,000	83,000	2,000	6
		second	20.75	25,000	4,500	2,500	2,000	3
	20/11	first	21.25	29,000	3,200	2,500	700	4
		second	21.50	31,000	420	390	30	5
7205	2/11	first	20.25	21,500	1,600	-	-	-
		second	22.00	36,000	3,200	-	-	-
	11/11	first	21.00	27,250	470	190	280	5
		second	21.75	33,500	260	240	20	4
	17/11	first	21.25	29,000	1,600	1,500	100	6
		second	22.75	44,500	110	0	110	3
8442	14/11	first	19.50	17,000	1,800	240	1,560	4
		second	20.50	23,250	28,000	27,900	100	6
72611	19/11	first	17.50	9,000	4,600	4,570	30	5
		second	16.50	6,400	1,300	700	600	3

/ . . .

APPENDIX TWO (contd)

BULL	DATE	EJACULATE NUMBER	ANTIBACTERIAL ACTIVITY		MICROBIAL POPULATION			
			Inhibition Zone (mm)	LzU_e/cm^3	Total Count	(a)[1]	(b)[2]	(c)[3]
72611 . .	22/11	first	17.75	9,600	770	530	240	3
		second	17.75	9,600	2,400	2,400	0	3
66175	16/11	first	21.50	31,000	320	82	238	3
		second	20.50	23,250	330	42	288	4

FOOTNOTE : [1] (a) = number of organisms resistant to the natural antibacterial activity of bovine semen.

[2] (b) = number of organisms sensitive to the natural antibacterial activity of bovine semen.

[3] (c) = number of different microbial colony types in each sample.

APPENDIX THREE

CHARACTERISTICS USED TO IDENTIFY THE BACTERIA ISOLATED FROM BOVINE SEMEN

These descriptions were compiled from data obtained using the media and techniques described in CHAPTER 6.

(i) Bacillus spp. These were aerobic, catalase positive (CHAPTER 6.2b), Gram-positive to Gram-variable (CHAPTER 6.2j)rods occurring singly or in chains. They produced central, ovoid to cylindrical spores, which did not distend the vegetative cells to any great extent. Colonies on blood agar medium (CHAPTER 6.1a) after 24 hours at 37'C were usually 1 - 2mm in diameter, circular to irregular, flat, butyrous, grey green and opaque. One colony, however, was rough, feathery, and sunken into the agar. Seven of the nine strains were alpha - haemolytic. Two produced pellicles on liquid medium, (CHAPTER 6.1b, 1c, 1d, 1e, 1f), while five grew with a characteristic floccular appearance.

The Bacillus spp. were heterotrophic, with light to medium growth in Davis & Mingioli salts medium (CHAPTER 6.2f), but with no growth in carbohydrate medium minus nitrogen compounds.

(ii) Corynebacterium spp. Species placed in this genus were all small, Gram-positive (CHAPTER 6.2j), slightly curved rods, some of which were slightly swollen at one end. They often occurred in a palisade or chinese letter arrangement and tended to adhere together when removed from the colony. Four of the isolates formed punctiform, flat, smooth, entire colonies with only light growth in 24 hours on blood agar (CHAPTER 6.1a). These colonies showed butyrous to slightly membranous, translucent to offwhite growth. The bacteria with this growth habit were nonmotile (CHAPTER 6.2n), catalase positive (CHAPTER 6.2b) and produced acid from glucose but not from fructose,galactose, lactose, maltose or salicin (CHAPTER 6.2c). The remaining four strains grew more readily on blood agar producing 1 - 2mm colonies in 24 hours. These colonies were circular, convex, smooth and moist with a butyrous texture. The bacteria were alpha - haemolytic giving a grey green appearance to colonies on blood agar plates. They also produced a strong odour. No acid or gas was produced from carbohydrates, but peptone water was strongly degraded with the resultant production of ammonia (CHAPTER 6.1f).

(iii) Micrococcus spp. These were Gram-positive (CHAPTER 6.2j), catalase positive (CHAPTER 6.2b), cocci, occurring singly, in pairs, in chains of three or four cells and in clusters. They were nonmotile, (CHAPTER 6.2n) coccal throughout their growth and reproduced by binary fission. Growth in liquid medium (CHAPTER 6.1b, 1c, 1d, 1e, 1f) was of uniform turbidity and colonies on blood agar (CHAPTER 6.1a) were smooth, circular and flat to convex in shape. Two strains grew relatively slowly at 37'C, producing slightly punctiform colonies of less than or about 1mm in diameter in 24 hours. Growth was opaque and butyrous, often with cream, yellow or orange pigmentation. All strains were able to grow on a defined medium with ammonium salts as the sole nitrogen source (CHAPTER 6.2f). An oxidation-fermentation tube test (CHAPTER 6.2i) indicated that they produced acid from glucose fermentatively. No acid was produced from fructose, galactose, lactose, maltose or salicin (CHAPTER 6.2c).

(iv) Pseudomonas spp. These were motile (CHAPTER 6.2n), Gram-negative (CHAPTER 6.2j) coccobacilli occurring singly, in pairs and occasionally in very short chains. On blood agar plates (CHAPTER 6.1a), they produced circular to irregular, smooth, convex colonies 1 - 2mm in diameter. These appeared moist and translucent to greyish white in colour, with a butyrous texture. They all produced a green water-soluble pigment which became brownish on storage at 4'C. Growth was aerobic, resulting in the slow production of acid from glucose (CHAPTER 6.2c) or the ammonification of peptone broth (CHAPTER 6.1f). They were catalase positive (CHAPTER 6.2b).

(v) Escherichia spp. Two isolates of motile (CHAPTER 6.2n), Gram-negative (CHAPTER 6.2j) coccobacilli, occurring singly, or in pairs and short chains were classified as Escherichia spp. They produced offwhite, translucent, circular, low convex, smooth colonies on blood agar (CHAPTER 6.1a) after 24 hours at 37'C. The growth was butyrous and had a distinct faecal odour. Culture on MacConkey agar (CHAPTER 6.21) yielded pink colonies, while that on Eosin Methylene Blue agar (CHAPTER 6.2g) produced dark purple colonies with darker centres and a metallic sheen. These bacteria were catalase positive (CHAPTER 6.2b), and produced acid and gas from fructose, galactose, glucose, lactose, and maltose (CHAPTER 6.2c). They were Methyl Red positive (CHAPTER 6.2m) and Voges-Proskauer negative (CHAPTER 6.2q).

(vi) Aerobacter spp. These were Gram-negative (CHAPTER6.2j) ,catalase positive (CHAPTER 6.2b), non motile (CHAPTER 6.2n) straight rods, some of which occurred singly, some in pairs or in short chains. They formed opaque, offwhite, circular, raised, smooth colonies with butyrous to mucoid growth on blood agar (CHAPTER 6.1a), light pink colonies on MacConkey agar (CHAPTER 6.21), and large pink mucoid colonies on Eosin Methylene Blue agar (CHAPTER 6.2g). The bacteria were Methyl Red negative (CHAPTER 6.2m), and Voges-Proskauer positive (CHAPTER 6.2q). Growth was aerobic and heterotrophic, resulting in the formation of acid and gas from fructose, galactose, glucose and maltose. Lactose was fermented slowly with only slight production of acid (CHAPTER 6.2c).

(vii) Alcaligenes sp. These were small, fine,Gram-negative (CHAPTER 6.2j) rods, occasionally occurring as filaments. On blood agar plates (CHAPTER 6.1a) they produced circular, flat to convex colonies, 1mm in diameter, with a smooth, entire, glistening to translucent appearance. Growth on MacConkey agar (CHAPTER 6.21) was colourless. The bacteria were catalase negative (CHAPTER 6.2b), urease negative (CHAPTER 6.2p), and able to grow in mineral salts medium (CHAPTER 6.2f).

(viii) Arthrobacter sp. This isolate showed a definite cycle of growth. In young peptone broth (CHAPTER 6.1f) culture,the cells were small, Gram-positive (CHAPTER 6.2j) cocci, irregular rods and a few filaments. After 12 hours, the predominant form was filamentous, with some rudimentary branching. Some of these filaments stained unevenly with methylene blue,showing the beginning of fragmentation. After approximately 48 hours, the fragmentation of the hyphae was well advanced, and soon after, coccoid forms predominated. These were often in chains,probably representing fragmented hyphae. In fresh medium, these cells developed into the rod-like forms seen in early cultures. Colonies on blood agar (CHAPTER 6.1a) were punctiform, flat to convex, rough and brittle. Growth was very light in 24 hours, with a dull white to orangy opaque appearance. On nutrient agar (CHAPTER 6.1d) at 30'C, the colonies were irregular

raised, rough to rugose, undulate to entire, and of opaque matt orange colouration. Growth in peptone broth (CHAPTER 6.1f) had a granular appearance with a pellicle on the surface. The bacteria were catalase positive (CHAPTER 6.2b), aerobic,and produced acid from fructose in carbohydrate utilisation tests (CHAPTER 6.2c). No acid or gas was produced from lactose. Litmus milk was darkened due to ammonification (CHAPTER 6.2k).

(ix) Brevibacterium sp. These cells were Gram-positive (CHAPTER 6.2j), short, pleomorphic rods with some tendency to chains or rod-like forms with swollen ends. There was some evidence of slime production. Culture on blood agar (CHAPTER 6.1a) yielded large (2mm or more), yellow, circular, convex, smooth colonies with glistening slightly mucoid growth. In peptone-meat extract broth (CHAPTER 6.1e), growth was of uniform turbidity, often resulting in an increased pH, due to ammonification. The bacteria were aerobic, catalase positive (CHAPTER 6.2b), oxidatively producing acid from glucose (CHAPTER 6.2i) but not from fructose. They were motile at 25'C (CHAPTER 6.2n), non cellulolytic (CHAPTER 6.2d), and hydrolysed gelatin (CHAPTER 6.2h). They did not affect litmus milk (CHAPTER 6.2k).

(x) Flavobacterium sp. These were Gram-negative (CHAPTER 6.2j) rods with slightly tapering ends,usually occurring singly. The colonies on blood agar (CHAPTER 6.1a) at 37'C after 24 hours were small (not more than 1mm in diameter), circular, flat, smooth, entire, opaque to translucent, and greyish yellow in colour. Growth in broth (CHAPTER 6.1b,1c,1d,1e &1f) was not uniformly turbid,but appeared to be slightly floccular. The bacteria were motile at 25'C (CHAPTER 6.2n) and catalase positive (CHAPTER 6.2b).

(xi) Microbacterium sp. These were Gram-variable (CHAPTER 6.2j), unevenly staining rods, slightly curved and often in pairs or groups. They produced punctiform, flat, smooth colonies with butyrous offwhite growth and less than 1mm in diameter after 24 hours on blood agar plates (CHAPTER 6.1a) at 37'C. A yellow grey colour appeared when the isolate was cultured on brain heart infusion agar (CHAPTER 6.1b). The bacteria were non motile (CHAPTER 6.2n), and catalase positive (CHAPTER 6.2b). They produced acid from glucose and fructose (CHAPTER 6.2c), and ammonia from peptone (CHAPTER 6.1f).

(xii) Nocardia sp. This isolate produced punctiform, raised, rough to rugose colonies with dryish brittle growth on blood agar (CHAPTER 6.1a) plates. They descended well into the agar,and the older regions became of greyish white appearance. When the organism was grown as a slide culture (CHAPTER 6.2o) , it was found that this isolate had a well developed mycellial stage, consisting of Gram-variable (CHAPTER 6.2j) filaments with distinct branching in the young hyphae. Both the aerial and the substrate mycellia fragmented to produce coccoid forms. No conidia were produced from the aerial hyphae by differentiation. The bacteria were non acid-fast (CHAPTER 6.2a), aerobic and catalase positive (CHAPTER 6.2b).

(xiii) Proteus sp. These were very small Gram-negative (CHAPTER 6.2j) rods,most of which occurred singly. They formed irregular,effuse, undulate,translucent, glistening colonies often swarming across the blood agar (CHAPTER 6.1a) plates. The bacteria were motile at 25'C and 37'C (CHAPTER 6.2n), urease positive (CHAPTER 6.2p), producing acid and gas from glucose, but no acid or gas from lactose (CHAPTER 6.2c). They were catalase positive (CHAPTER 6.2b).

(xiv) Sarcina sp. The isolate classified as Sarcina (Brown et al 1974), consisted of Gram-positive (CHAPTER 6.2j), catalase positive

(CHAPTER 6.2b) cocci, occurring in a cubical packet arrangement. They produced circular, convex,matt colonies with an entire edge, opaque yellow in colour with butyrous growth, 1 - 2mm in diameter after 24 hours at 37'C on blood agar (CHAPTER 6.1a) plates. These bacteria were aerobic, producing acid oxidatively from glucose (CHAPTER 6.2c). In the eighth edition of Bergey's Manual of Determinative Bacteriology (1974), the genus Sarcina has been restricted to anaerobic cocci, and this isolate is currently classified as Micrococcus sp.

(xv) Staphylococcus sp. These bacteria were non motile (CHAPTER 6.2n), Gram-positive (CHAPTER 6.2j), catalase positive (CHAPTER 6.2b) cocci occurring singly, in pairs, in chains and clusters. The colonies they produced on blood agar (CHAPTER 6.1a) after 24 hours at 37'C were circular, convex, smooth and entire with butyrous creamy growth approximately 1mm in diameter. The organisms were aerobic or faculatative anaerobes able to produce acid but not gas from glucose by fermentation (CHAPTER 6.2i). They were unable to utilise ammonium salts as their sole nitrogen source (CHAPTER 6.2f).

In addition to the bacteria described above, two isolates were identified as Herellea spp.[1] , by Dr B.D.W. Jarvis , Department of Microbiology and Genetics,Massey University, Palmerston North, New Zealand.

The remaining two isolates were yeasts, which were subsequently identified as Rhodotorula rubra and Candida guilliermondii . These identifications were made by Dr M. Baxter of the same department.

FOOTNOTE: [1] Currently classified as Acinetobacter , (Bergey 1974)

APPENDIX FOUR

THE RESISTANCE OF SEMEN MICROORGANISMS TO COMMERCIAL ANTIBIOTICS AND TO THE INHIBITORY ACTIVITY OF BOVINE SEMINAL PLASMA.

BACTERIUM[1]	NUMBER OF STRAINS RESISTANT TO:												
	SP[2]	(A)[3]				(B)[4]			(C)[5]					(D)[6]
		BAC	CYC	PEN	RIS	COL	POL	NYS	CHL	ERY	KAN	SM	TET	SUL
Aerobacter (2)	0	2	1	2	2	0	0	2	0	2	0	0	0	2
Alcaligenes (1)	1	1	1	1	1	0	0	1	0	0	0	0	0	1
Arthrobacter (1)	0	0	0	0	0	1	1	1	0	0	0	0	0	1
Bacillus (9)	2	2	4	3	0	1	1	8	0	0	1	0	0	4
Candida (1)	1	1	1	1	1	1	1	0	1	1	1	1	1	1
Corynebacterium (7)	3	2	6	3	3	3	2	7	0	3	1	0	0	4
Escherichia (2)	0	2	1	2	2	0	0	2	0	2	0	1	1	2
Microbacterium (1)	0	0	1	0	0	0	1	1	0	0	0	0	0	1
Micrococcus (6)	0	0	4	1	0	1	1	6	0	0	0	1	2	3
Nocardia (1)	0	0	1	1	0	0	0	1	0	0	0	0	0	1
Proteus (1)	1	1	1	1	1	1	1	1	0	1	0	0	0	1
Pseudomonas (4)	4	4	4	4	4	0	0	3	4	4	2	0	4	4
Rhodotorula (1)	1	1	1	1	1	1	0	0	1	1	1	1	1	1
Sarcina*(1)	0	0	1	0	0	0	0	1	0	0	0	0	0	1
Staphylococcus (1)	1	0	0	0	0	0	1	1	0	0	0	0	0	1

FOOTNOTES:
[1] number of strains tested in parentheses .
[*] currently classified as Micrococcus (Bergey 1974).
[2] SP = bovine seminal plasma.
[3] (A) = antibiotics affecting cell wall synthesis; BAC= bacitracin, CYC= cycloserine, PEN= penicillin, RIS= ristocetin.
[4] (B) = antibiotics affecting cell membrane integrity; COL= colistin sulphate, POL= polymyxin, NYS= nystatin.
[5] (C) = antibiotics affecting protein synthesis; CHL= chloramphenicol, ERY= erythromycin, KAN= kanamycin, SM= streptomycin, TET= tetracycline.
[6] (D) = metabolic analogue antibiotic; SUL= sulfafurazole.

APPENDIX FIVE

SENSITIVITY OF A VARIETY OF BACTERIA TO BOVINE SEMINAL PLASMA, HUMAN SEMINAL PLASMA, LYSOZYME, POLYLYSINE, SALMINE AND SPERMIDINE.

GRAM POSITIVE BACTERIA	ZONE DIAMETER (mm) IN RESPONSE TO:					
	Bovine Seminal Plasma.	Human Seminal Plasma	Lysozyme	Polylysine	Salmine	Spermidine
Arthrobacter globiformis	25.00	24.00	29.00	30.50	27.00	32.50
Bacillus cereus	15.00	10.50	20.50	20.50	17.50	16.50
Bacillus licheniformis	12.50	0	21.00	21.25	18.75	0
Bacillus megaterium	12.00	0	0	16.75	15.50	0
Bacillus megaterium (asporing)	21.00	25.00	21.00	24.00	18.50	26.50
Bacillus mycoides	13.00	0	0	19.00	16.50	0
Bacillus mycoides	12.50	0	10.50	21.00	18.00	16.50
Bacillus stearothermophilus	15.00	10.75	20.50	19.50	17.00	0
Bacillus subtilis	12.00	0	0	16.50	15.50	0
Corynebacterium diphtheriae v.gravis	14.50	0	0	18.00	17.50	0
Corynebacterium diphtheriae v.intermedius	13.50	0	0	22.50	21.00	18.00
Corynebacterium diphtheriae v.mitis	15.00	0	0	21.75	19.00	0
Corynebacterium hofmanni	18.50	0	0	26.25	23.00	18.00
Corynebacterium pyogenes*	11.00	26.50	15.50	13.25	12.00	29.00
Corynebacterium xerosis*	18.00	0	11.50	24.50	23.50	29.50
Lactobacillus bulgaricus*	0	0	0	20.50	18.00	31.00
Lactobacillus bulgaricus*	17.50	16.50	23.50	16.25	15.50	13.00
Lactobacillus casei*	0	13.50	0	28.00	24.00	0
Lactobacillus casei v.alactosus*	12.50	14.00	0	19.50	16.50	0
Lactobacillus casei v.rhamnosus*	11.75	0	11.00	18.50	16.50	0
Lactobacillus plantarum*	13.50	18.50	13.00	16.00	14.25	0
Leuconostoc citrovorum*	10.50	16.50	0	19.50	16.75	26.50
Leuconostoc mesenteroides*	12.00	11.00	16.00	23.00	19.25	0
Micrococcus luteus	22.00	21.75	37.00	28.00	25.00	14.50
Micrococcus lysodeikticus	20.50	18.25	29.50	28.50	23.00	11.00
Micrococcus lysodeikticus*	21.00	21.00	32.50	24.25	22.50	29.00
Micrococcus lysodeikticus#	18.00	19.00	28.00	26.75	23.25	0

/ . . .

APPENDIX FIVE (contd)

GRAM POSITIVE BACTERIA	ZONE DIAMETER (mm) IN RESPONSE TO:					
	Bovine Seminal Plasma	Human Seminal Plasma	Lysozyme	Polylysine	Salmine	Spermidine
Mycobacterium phlei*	10.50	19.00	27.75	26.00	25.00	28.25
Nocardia asteroides	0	0	0	0	19.50	0
Sarcina lutea	15.75	16.75	26.75	19.50	19.25	28.50
Staphylococcus aureus	11.25	12.50	0	17.75	14.50	23.00
Staphylococcus aureus	14.00	13.00	0	17.50	15.00	23.50
Staphylococcus aureus	12.00	0	0	17.00	15.25	0
Staphylococcus aureus	11.00	0	0	17.50	15.50	26.00
Staphylococcus aureus	13.00	15.00	0	18.00	15.00	0
Staphylococcus aureus	0	10.50	0	17.00	13.50	17.50
Staphylococcus aureus	11.00	14.00	0	16.50	14.75	20.50
Staphylococcus aureus	12.00	13.25	0	17.00	15.00	21.75
Staphylococcus epidermidis	12.50	18.50	0	18.75	16.75	25.00
Staphylococcus epidermidis	10.75	0	18.00	21.50	17.50	0
Staphylococcus epidermidis	12.00	14.00	0	18.50	15.25	26.00
Streptococcus cremoris*	36.00	26.00	0	0	0	0
Streptococcus equi*	12.00	0	0	22.00	18.00	32.00
Streptococcus faecalis	13.00	0	23.50	14.50	13.50	0
Streptococcus faecalis*	0	10.50	0	18.25	17.50	11.00
Streptococcus faecalis*	0	10.50	0	17.00	14.75	0
Streptococcus lactis*	26.75	22.00	0	0	0	0
Streptococcus mitis*	0	0	11.00	14.50	15.50	0
Streptococcus mitis*	11.50	11.00	0	17.00	15.25	11.75
Streptococcus mutens*	0	0	0	15.50	14.75	0
Streptococcus pneumoniae	0	0	12.50	19.50	17.00	0
Streptococcus pyogenes*	14.50	12.50	0	13.00	16.50	11.50
Streptococcus salivaris*	19.50	15.25	16.00	18.75	18.50	42.00
Streptomyces sp.	21.00	13.00	24.50	30.00	22.50	12.50
GRAM NEGATIVE BACTERIA						
Acetobacter sp.	11.00	11.00	0	17.50	14.50	0
Citrobacter freundii	14.50	0	0	15.00	12.50	0

/ . . .

APPENDIX FIVE (contd)

GRAM NEGATIVE BACTERIA	ZONE DIAMETER (mm) IN RESPONSE TO:					
	Bovine Seminal Plasma	Human Seminal Plasma	Lysozyme	Polylysine	Salmine	Spermidine
Enterobacter cloacae	12.00	0	0	0	11.50	0
Escherichia coli Ø P1	16.75	14.00	15.00	16.75	15.75	0
Escherichia coli B	17.00	11.50	0	17.50	16.00	0
Escherichia coli B	15.50	15.50	14.50	17.00	14.50	0
Escherichia coli B	15.50	12.25	14.75	16.00	14.50	0
Escherichia coli B	17.00	15.00	15.25	16.00	15.00	0
Escherichia coli C	18.50	14.50	15.00	17.00	15.00	0
Escherichia coli K 12	16.00	14.50	0	17.25	14.75	0
Escherichia coli W	15.75	13.50	0	17.00	15.00	0
Escherichia dispar	15.00	17.50	0	17.00	14.25	0
Haemophilus sp.#	11.50	12.50	19.50	23.00	20.50	18.00
Klebsiella sp.	13.00	0	0	11.50	13.00	0
Klebsiella aerogenes	12.00	0	0	0	12.00	0
Klebsiella edwardsii v.edwardsii	16.00	0	0	18.00	16.50	0
Neisseria flavescens*	32.00	34.50	24.00	22.00	21.00	48.00
Neisseria gonorrhoea*	11.50	26.75	17.50	14.25	14.25	38.00
Neisseria meningitidis*	16.50	25.00	14.50	13.50	16.50	35.00
Pasteurella haemolytica*	12.00	12.50	0	21.50	19.50	26.50
Proteus mirabilis	0	0	0	13.25	12.00	0
Proteus morganii	0	0	0	0	13.50	0
Proteus rettgeri	0	0	0	0	11.50	0
Proteus vulgaris	0	0	12.00	12.00	11.00	0
Proteus vulgaris	0	0	13.00	12.25	13.50	0
Providence sp.	0	0	0	18.00	15.00	0
Pseudomonas aeruginosa	0	0	0	17.00	14.75	0
Pseudomonas fluorescens	17.25	0	11.00	21.00	17.25	0
Salmonella arizonae	14.00	0	0	16.00	15.00	0
Salmonella cholerae suis	14.50	13.50	0	18.00	16.50	0
Salmonella gallinarum	17.00	0	0	19.50	16.50	0
Salmonella newington	12.75	0	0	13.00	13.75	0
Salmonella typhimurium	13.00	0	0	14.00	13.50	0

/ . . .

APPENDIX FIVE (contd)

GRAM NEGATIVE BACTERIA	ZONE DIAMETER (mm) IN RESPONSE TO:					
	Bovine Seminal Plasma	Human Seminal Plasma	Lysozyme	Polylysine	Salmine	Spermidine
Salmonella typhimurium	13.25	12.00	0	14.00	13.75	0
Serratia marcescens	13.50	0	0	11.00	11.50	0
Shigella flexneri	16.50	18.25	11.50	18.25	15.75	28.00
Shigella sonnei	16.50	17.25	14.00	17.50	14.75	0
Vibrio anguillarum*	14.75	11.75	19.00	18.50	16.25	16.50

FOOTNOTES: * medium supplemented with 10% serum.
medium supplemented with haemin and NADH.

BIBLIOGRAPHY.

ABRAMOVA,L.N. ; OBOLADZE,D.B. ; TERSKIKH,I.I. 1974 : Characteristics of Chlamydiae isolated from cattle. Voprosy Virusologii 6 : 737 - 9. Veterinary Bulletin 45 (1975) abstract 3728.

ADLER,H.C. 1965 : Bovine genital vibriosis. Nordisk Veterinaermedicin17:237-41.

ADLER,H.C. 1966 : Bovine genital vibriosis : transmission through deep-frozen semen and its prevention by streptomycin treatment. Aarsberetning. Inst. Sterlitetsforsk., Copenhagen (1966) pp 149 - 54. In Danish. Veterinary Bulletin 37 (1967) abstract 1093.

ADLER,H.C. ; ANDERSEN,J.B. 1970 : Method for the inactivation of virus in semen straws. Acta Veterinaria Scandinavica 11 : 484 - 6.

AFSHAR,A. 1965 : Virus diseases associated with bovine abortion and infertility. Veterinary Bulletin 35 : 735 - 52.

AL-AUBAIDI,J.M. ; McENTEE,K. ; LEIN,D.H. ; ROBERTS,S.J. 1972 : Bovine seminal vesiculitis and epididymitis caused by Mycoplasma bovigenitalium . The Cornell Veterinarian 62 : 581 - 96.

ALMQUIST,J.O. 1948 : The effect of penicillin upon the fertility of semen from relatively infertile bulls. Journal of Dairy Science 31 : 681 - 2.

ALMQUIST,J.O. 1949a : A comparison of penicillin, streptomycin and sulfanilamide for improving the fertility of semen from relatively infertile bulls. Journal of Dairy Science 32 : 722 (abstract).

ALMQUIST,J.O. 1949b : The effect of penicillin upon the fertility of semen from relatively infertile bulls. Journal of Dairy Science 32 : 950 - 4.

ALMQUIST,J.O. 1951 : A comparison of penicillin, streptomycin and sulfanilamide for improving the fertility of semen from bulls of low fertility. Journal of Dairy Science 34 : 819 - 22.

ALMQUIST ,J.O. ; PRINCE,P.W. 1950 : The Effect of a combination of penicillin and streptomycin and sulfanilamide upon the fertility of bull semen. Journal of Dairy Science 33 : 393 - 4.

ALMQUIST,J.O. ; THORP,W.T.S. ; KNODT,C.B. 1946 : The effect of penicillin upon the liveability, fertility and bacterial content of bull semen. Journal of Animal Science 5 : 400 - 1.

ALMQUIST,J.O. ; GLANTZ,P.J. ; SHAFFER,H.E. 1949a : The effect of a combination of penicillin and streptomycin upon the liveability and bacterial content of bovine semen . Journal of Dairy Science 32 : 183 - 90.

ALMQUIST,J.O. ; PRINCE,P.W. ; REID,J.J. 1949b : Bacteriological studies of bovine semen 1. Numbers of bacteria and the relation to fertility. Journal of Dairy Science 32 : 543 - 8.

AMATREDJO,A. ; CAMPBELL,R.S.F. 1975 : Bovine leptospirosis. Review in Veterinary Bulletin 45 : 875 - 91.

AWAD,F.I. 1960 : Nocardiosis of the bovine udder and testes Veterinary Record 72 : 341 - 2.

BAILEY,N.T.J. 1968 : "Statistical Methods in Biology ". English University Press Ltd., London,England.

BAGSHAW,P.A. ; LADDS,P.W. 1974a : Pathology of the accessory sex glands of the bull. Review in Veterinary Bulletin 44 : 343 - 8.

BAGSHAW,P.A. ; LADDS,P.W. 1974b : A study of the accessory sex glands of bulls in abbatoirs in North Australia. Australian Veterinary Journal 50 : 489 - 95.

BERGEY, 1974 : " Bergey's Manual of Determinative Bacteriology ". 8th edition. R.E. Buchanan & N.E. Gibbons (editors) Williams & Wilkins Co., Baltimore, U.S.A.

BITSCH,V. 1964 : Infectious bovine rhinotracheitis virus infection in bulls, with special reference to preputial infection. Applied Microbiology 26 : 337 - 43.

BLAIR,J.E. ; LENNETTE,E.H. ; TRUANT,J.P. 1970 : "Manual of Clinical Microbiology ". American Society for Microbiology,Bethesda,Maryland 21202, U.S.A.

BLOM,E. ; DAM,A. 1967 : Occurrence of Escherichia coli in semen from Danish A.I. bulls observed during two years. Meddr. St. Vet. Serumlab. No 438. In Danish. Veterinary Bulletin 37 : abstract 4056.

BLOM,E. ; ERNO,H. 1967 : Mycoplasmosis,infections of the genital organs of bulls. Acta Veterinaria Scandinavica 8 : 186 - 8.

BLOM,E. ; ERNO,H. ; 1970 :Mycoplasmas in semen of Danish breeding bulls. Proc. 11th Nordic Vet. Congress,Bergen 1970, p354. Veterinary Bulletin 41 (1971) abstract 1668.

BORYCZKO,Z. ; SADOWSKI,J.M. ; TRUSZCZYNSKI,M. ; MAJCHRZYK,H. 1973 : Outbreak of Bedsonia infection in a herd of bulls, Medycyna Weterynaryjna 29 : 483 - 5.

BOTTCHER,R. ; MAHLER,R. 1970 : Investigations on the persistence of the bovine IBR/IPV virus in the semen of naturally infected bulls. Deutsche Tierarztliche Wochenschrift 77 : 421 - 4. In German, English summary.

BOUTERS,R. 1964 : A virus with enterogenic properties causing degeneration of the germinal epithelium in bulls. Nature,London 201 : 217 - 8.

BRANNY,J. ; ZEMBALA,M. 1971 : Some characteristics of viruses isolated from bull semen and their possible pathogenicity. British Veterinary Journal 127 : 88 - 92.

BRANNY,J. ; ZGORNIAK- NOWOSIELSKA,I. 1971 : Mycoplasma in bull semen II . Serological properties of mycoplasma in bull semen. Medycyna Weterynarvina 27 : 113 - 5 . In Polish. Biological Abstracts 53 (1972) : abstract 50046.

BROWN,V.G. ; SCHOLLUM,L.M. ; JARVIS,B.D.W. 1974 : Microbiology of bovine semen and artificial breeding practices under New Zealand conditions. New Zealand Journal of Agricultural Research 17 : 431 - 42.

BROWNLIE,J. 1971 : Antimicrobial proteins in bovine neutrophils. Biochemical Journal 125 : 81p - 82p .

BROWNLIE,J. ; HIBBITT,K.G. 1972 : Antimicrobial proteins isolated from bovine cervical mucus. Journal of Reproduction and Fertility 29 : 337 - 47.

BRUNE,K. ; SPITZNAGEL,J.K. 1973 : Peroxidaseless chicken leukocytes ; isolation and characterisation of antibacterial granules. The Journal of Infectious Diseases 127 : 84 - 94.

BULLEN,J.J. ; ROGERS,H.J. ; LEIGH,L. 1972 : Iron-binding proteins in milk and resistance to Escherichia coli infection in infants. British Medical Journal 1 : 69 - 75.

BURGESS,G.W. ; CHENOWETH,P.J. 1975 : Midpiece abnormalities in bovine semen following experimental and natural cases of bovine ephemeral fever. British Veterinary Journal 131 : 536 - 44.

BUSCH,H. 1965 : " Histones and other nuclear Proteins ". Academic Press, New York, U.S.A.

BWANGAMOI,O. ; KAMINJOLO,J.S. 1971 : Isolation of IBR/IPV virus from the semen and skin lesions of bulls at Kabete,Kenya. Zentralblatt für Veterinarmedizin, Reihe B 18 : 262 - 9.

CHANDAN,R.C. ; PARRY,R.M. ; SHAHANI,K.M. 1968 : Lysozyme, lipase and ribonuclease in various species. Journal of Dairy Science 51 : 606 - 7.

CLARKE,J.K. 1976 : Personal communication. Department of Microbiology and Genetics, Massey University, Palmerston North. New Zealand.

CLARK,B.L. ; MONSBOURGH,M.J. ; DUFTY,J.H. 1974a : Isolation of Campylobacter (Vibrio) fetus subspecies venerealis and Campylobacter fetus subspecies intermedius from preputial secretions of bulls. Australian Veterinary Journal 50 : 324.

CLARK,B.L. ; PARSONSON,I.M. ; DUFTY,J.H. 1974b : Experimental infection of bulls with Trichomonas fetus. Australian Veterinary Journal 50 : 189-91.

COLOWICK,S.P. ; KAPLAN,N.O. 1973 : (editors-in-chief) " Methods in Enzymology". volume xxvii. ' Enzyme Structure,part D ' edited by C.H.W. Hirs & S.N. Timasheff. Academic Press Inc.,New York and London.

CONWAY,E.J. 1957 : " Microdiffusion Analysis and Volumetric Errors ",p 108. 4th revised edition. Crosby, Lockwood & son Ltd.,London, England.

COTTRAL,G.E. ; GAILIUNAS,P. ; COX,B.F. 1968 : Foot-and-mouth Virus in semen of bulls and its transmission by artificial insemination. Archiv für die Gesamte virusforschung 23 : 362 - 77 . English summary.

CRUICKSHANK,R. 1969 : " Medical Microbiology,a guide to the laboratory diagnosis and control of infection." 11th edition,revised reprint. E. & S. Livingstone Ltd., Edinburgh and London, Great Britain.

DAVIES,D.C. ; HALL,G. ; HIBBITT,K.G. ; MOORE,H.D.M. 1975 : The removal of the seminal vesicles from the boar and the effects on the semen characteristics. Journal of Reproduction and Fertility 43 : 305 - 12.

DAVIS,W.W. ; STOUT,T.R. 1971 : Disc plate method of microbiological antibiotic assay 1. factors influencing variability and error.
2. novel procedure offering improved accuracy. Applied Microbiology 22 : 659 - 70.

DAWSON,R.M.C. ; ELLIOTT,D.C. ; ELLIOTT,W.H. ; JONES,K.M. 1969 : Editors of " Data for Biochemical Research ". 2nd edition. Oxford University Press, London, England.

DE,B.N. ; CHOWDHURY,T.M. 1965 : Bacterial flora of bovine semen . Indian Journal of Animal Health 4 : 27 - 31. Biological Abstracts 48 (1967) abstract 113063.

DEAS,D.W. ; JOHNSTON,W.S. ; VANTSIS,J.T. 1966 : The isolation of para-influenza III virus from the testicles of an infertile bull. Veterinary Record 78 : 739 - 40.

DENNETT,D.P. ; ALLAN,P.J. ; JOHNSON,R,H, 1973 : The use of corticosteroids to aid detection of bulls carrying IBR virus. Australian Veterinary Journal 49 : 594 - 5.

DENT,G.H.R. 1971 : Ulcerative vulvitis and posthitis in Australian sheep and cattle. Veterinary Bulletin 41 : 719 - 23.

DONALDSON,L.E. ; AUBREY,J.N. 1960 : Posthitis and prolapse of the prepuce in cattle. Australian Veterinary Journal 36 : 380 - 3.

DUNN,H.O. ; BURDA,K. ; WAGNER,W.C. ; GILMAN,H.L. 1965 : Isolation of Vibrio fetus from bovine semen. Cornell Veterinarian 55 : 220 - 9.

EASTERBROOKS,H.L. ; HELLER,P. ; PLASTRIDGE ,W.N. ; JUNGHERR,E.L. ; ELLIOT,F.I. 1950 : The fertility of bull semen containing 100 micrograms of streptomycin per millilitre. Journal of Dairy Science 33 : 737 - 40.

EDMONDSON,J.E. ; TALLMAN,K.L. ; HERMAN,H.A. 1948 : The study of the types of bacteria in bovine semen and their effect upon motility. (abstract). Journal of Dairy Science 31 : 681.

EISELE,K. 1975 : Antibiotically active tripeptides of the sequence larg-Dx-Lphe. Experentia 31 : 764 - 5. English summary.

ERB,R.E. ; FLERCHINGER,F.H. 1954 : The influence of fertility level and treatment of semen on non-return decline for 29 to 180 days following artificial service. Journal of Dairy Science 37 : 938 - 48.

ERN,H. ; PLASTRIDGE,W.N. ; TOURTELLOTTE,M.E. 1967 : Mycoplasma; isolation from prepuce and semen of bulls. Acta Veterinaria Scandinavica 8 : 123 - 35.

FAIR,W.R. ; WEHNER,N. 1971a : Antibacterial action of spermine : Effect on urinary tract pathogens. Applied Microbiology 21 : 6 - 8.

FAIR,W.R. ; WEHNER,N. 1971b : Further observations on the antibacterial nature of prostatic fluid. Infection and Immunity 3 : 494 - 5.

FITZGERALD,P.R. ; HAMMOND,D.M. ; MINER,M.L. 1954 : Survival and transmission of Trichomonas fetus in diluted bovine semen. American Journal of Veterinary Research 15 : 36 - 40.

FLERCHINGER,F.H. ; ERB,R.E. 1953 : Service sire influences on fertility rates of repeat breedings. Journal of Dairy Science 36 : 1072 - 6.

FOOTE,R.H. ; BRATTON,R.W. 1950a : The fertility of bovine semen in extenders containing sulfanilamide, penicillin, streptomycin and polymyxin. Journal of Dairy Science 33 : 544 - 547.

FOOTE,R.H. ; BRATTON,R.W. 1950b : Motility of bovine spermatozoa and control of bacteria at 5'C and 25'C in extenders containing sulphanilamide, penicillin, streptomycin and polymyxin. Journal of Dairy Science 33 : 842 - 6.

FOOTE,R.H. ; BRATTON,R.W. 1952 : The influence of antibiotics on delayed returns in artificial breeding. Journal of Dairy Science 35 : 261 - 5.

FOOTE,R.H. ; SALISBURY,G.W. 1948 : The effect of sulphonamides upon the liveability of spermatozoa and upon the control of bacteria in diluted bull semen. Journal of Dairy Science 31 : 769 - 78.

FREY,M.L. ; HANSON,R.P. ; ANDERSON,D.P. 1968 : A medium for the isolation of avian mycoplasmas. American Journal of Veterinary Research 29 : 2163 - 71.

GALLOWAY,D.B. 1964 : A study of bulls with the clinical signs of seminal vesiculitis; clinical, bacteriological and pathological aspects. Acta Veterinaria Scandinavica 5 : supplementum 2.

GETTY,S.M. ; EILIS,D.J. 1967 : The experimental use of bull semen contaminated with Pseudomonas aeruginosa organisms. Journal of American Veterinary Medicine Association 151 : 1688 - 91.

GIBBONS,R.A. 1959 : Chemical properties of two mucoids from bovine cervical mucus. Biochemical Journal 73 : 209 - 17.

GIBBONS,W.J. 1963 : "Diseases of Cattle " . American Veterinary Publications Inc., U.S.A.

GILMAN,H.L. 1921 : A study of some factors influencing fertility and sterility in the bull. Report of the New York Veterinary College, p68 - 126. Cited by Gunsalus et al (1941).

GILMAN,H.L. ; HOPPER,E.B. 1925 : An interesting case of sterility in the bull. Cornell Veterinarian 15 : 333 - 7.

GLADSTONE,G.P. ; WALTON,E. 1971 : Effect of iron and haematin on the killing of staphylococci by rabbit polymorphs. British Journal of Experimental Pathology 52 : 452 - 64.

GROSSOWICZ,N. ; RAZIN,S. ; ROZANSKY,R. 1955 : Factors influencing the antibacterial action of spermine and spermidine on Staphylococcus aureus. Journal of General Microbiology 13 : 436 - 41.

GUNSALUS,I.C. ; SALISBURY,G.W. ; WILLETT,E.L. 1941 : The bacteriology of bull semen . Journal of Dairy Science 24 : 911 - 9.

GUNSALUS ,I.C. ; CAMPBELL,J.J. ; BECK,G.H. ; SALISBURY,G.W. 1944 : The bacteriology of bull semen II. The effect of bacteria upon rapid tests in semen quality. Journal of Dairy Science 27 : 357 - 64.

GUREVITCH,J. ; ROZANSKY,R. ; WEBBER,D. ; BRZEZINSKY,A. ; ECKERLING,B. 1951 : The role of spermine in the inhibition of Staphylococcus aureus by human semen. Journal of Clinical Pathology 4 : 360 - 5.

HAMDY,A.H. ; MILLER,C.C. 1971 : Antibiotics for bovine mycoplasmas. Journal of Dairy Science 54 : 1541 - 4.

HANKIEWICZ,J. ; SWIERCZEK,E. 1974 : Lysozyme in human body fluids. Clinica Chimica Acta 57 : 205 - 9.

HAROLD,F.M. 1970 : Antimicrobial agents and membrane function. Advances in Microbial Physiology 4 : 46 -104.

HASHIMOTO,K. 1963 : Studies on the control of bacterial contamination of bull semen 1. Bacterial contamination of undiluted semen and diluted semen treated with antibiotics. Bulletin of the National Institute of Animal Health 46 : 36 - 44.

HATZIOLOS,B. 1937 : Untersuchunger über Konservierung von Bullensperma zum Zwecke der künstlichen Besamung. Zeitschrift für Züchtung, Reihe B : Tierzuchtung und Zuchtungsbiologie 38 : 199 - 254 . Cited by Gunsalus et al (1941).

HEFTMANN,E. 1967 : " Chromatography ". 2nd edition. Reinhold Publishing Corp., New York, U.S.A.

HENDRIKSE,J. 1963 : Bacterial content of diluted and stored semen. Tijdschr. Diergeneesk 88 : 506 - 9. Veterinary Bulletin 33 :abstract 3361.

HIBBITT,K.G. ; BENIANS,M. 1971 : Some effects in vivo of the teat canal and in vitro of cationic proteins on Staphylococci. Journal of General Microbiology 68 : 123 - 8.

HIBBITT,K.G. ; COLE,C.B. 1968 : The antimicrobial activity of teat canal cationic proteins. Biochemical Journal 106 : 39p.

HIBBITT,K.G. ; COLE,C.B. ; REITER,B. 1969 : Antimicrobial proteins isolated from the teat canal of the cow. Journal of General Microbiology 56 : 365 - 71.

HIBBITT,K.G. ; BROWNLIE,J. ; COLE,C.B. 1971 : The antimicrobial activity of cationic proteins isolated from the cells in bulk milk samples. Journal of Hygiene, Cambridge 69 : 61 - 8.

HIBBITT,K.G. ; BENIANS,M. ; ROWLANDS,G.J. 1972 : The number and percentage viability of commensal microorganisms recovered from the teat canal of the cow. British Veterinary Journal 128 : 270 - 4.

HILL,A.W. ; HIBBITT,K.G. ; SHEARS,A. 1973 : The stimulation by endotoxin of the non specific resistance of mice to bacterial infection. British Journal of Experimental Pathology 55 : 194 - 202.

HIRSCH,J.G. 1958 : The bacteriocidal action of histones. Journal of Experimental Medicine 108 : 925 - 44.

HIRTH,R.S. ; NIELSEN,S.W. 1966 : Experimental pathology of bovine salpingitis due to mycoplasma insemination. Laboratory Investigation 15 : 1132 - 5.

HIRTH,R.S. : NIELSEN,S.W. ; PLASTRIDGE,W.N. 1966 : Bovine salpingo - oophoritis produced with semen containing a mycoplasma. Pathologica Veterinaria 3 : 616 - 32.

HIRTH,R.S. ; PLASTRIDGE,W.N. ; TOURETELLOTTE,M.E. 1967 : Survival of a mycoplasma in frozen bovine semen. American Journal of Veterinary Research 28 : 97 - 9.

HOLT,A.F. 1952 : The effect of sulfanilamide on the fertility of bull semen. Veterinary Record 64 : 93 - 4.

HOWARD,C.J. ; BROWNLIE,J. ; GOURLAY,R.N. ; COLLINS,J. 1975 : Presence of a dialysable fraction in normal bovine whey capable of killing several bovine mycoplasmas . Journal of Hygiene, Cambridge 74 : 261 - 70.

HUGH- JONES,M.E. ; AUSTWICK,P.K.C. 1967 : Epidemiological studies in bovine mycotic abortion 1. the effect of climate on incidence. Veterinary Record 81 : 273 - 6.

HUMPHREY,J.D. ; LADDS,P.W. 1975 : Pathology of bovine testes and epididymis. Review in Veterinary Bulletin 45 : 787 - 97.

JASPER,D.E. 1967 : Mycoplasmas ; their role in bovine disease. Journal of the American Veterinary Medicine Association 151 : 1650 - 5 .

JASPER,D.E. ; AL-AUBAIDI,J.M. ; FABRICANT,J. 1974 : Isolation of Mycoplasmas from preputial washings of bulls. Cornell Veterinarian 64 : 296 - 302.

JOHNSTON,W.S. ; DEAS,D.W. 1971 : Isolation of a paravaccinia virus from bovine semen. Veterinary Record 89 : 450.

JONES,T.H. ; BARRETT,K.J. ; GREENHAM,L.W. ; OSBORNE,A.D. ; ASHDOWN,R.R. 1964 : Seminal vesiculitis in bulls associated with infection by Actinobacillus actinoides. Veterinary Record : 76 : 74 - 8.

JOYNER,L.P. ; MILLER,P.G. 1952 : Transmission of Trichomonas fetus during collection of semen from infected bulls. Veterinary Record 64 : 1 - 4.

KAMINJOLO,J.S. ; NYAGA,P.N. ; OMUSE,J.K. ; MUTIGA,E.R. 1975 : Infectious bovine rhinotracheitis- infectious pustular vulvovaginitis viral isolates from cattle with epididymitis and vaginitis. American Journal of Veterinary Research 36 : 123 - 5.

KAZDA,J. 1963 : The flora of the genital organs and semen of bulls of low fertility. Veterinari medicina, Prague 8 : 317 - 24. Veterinary Bulletin 34 : abstract 1135.

KENDRICK,J.W. ; McENTEE,K. 1967 : The effect of artificial insemination with semen contaminated with IBR/IPV (infectious bovine rhinotracheitis / infectious pustular vulvovaginitis) virus. Cornell Veterinarian 57 : 3 - 11.

KIMBALL,A. ; TWIEHAUS,M.J. ; FRANK,E.R. 1954 : Actinomyces bovis isolated from six cases of bovine orchitis. A preliminary Report. American Journal of Veterinary Research 15 : 551 - 3.

KNOP,J. ; ROWLEY,D. 1975 : Protection against cholera, an antibacterial mechanism on the mucosal surface of the small intestine of mice. Australian Journal of Experimental Biology and Medical Science 53 : 155 - 65.

LADDS,P.W. ; DENNETT,D.P. ; GLAZEBROOK,J.S. 1973 : A survey of the genitalia of bulls in North Australia. Australian Veterinary Journal 49 : 335 -40.

LANGFORD,E.V. 1974 : Mycoplasma infections in the bovine genital tract. Canadian Veterinary Journal 15 : 300 - 1.

LAGERLOF,N. 1936 : Sterility in bulls. Veterinary Record 47 : 1159 - 73.

LARSEN,A.B. ; KOPECKY,K.E. 1970 : Mycobacterium paratuberculosis in reproductive organs and semen of bulls. American Journal of Veterinary Research 31 : 255 - 8.

LE Q. DARCEL,C. ; COULTER,G.H. 1976 : IBR neutralising substance in bull seminal fluid and its removal prior to attempts at virus isolation from semen. Canadian Veterinary Journal 17 : 318 - 20.

LEHRER,R.I. 1973 : The bacteriocidal components of human neutrophil (PMN) granules. Clinical Research 21 : 560.

LEHRER,R.I. 1975 : The fungicidal mechanisms of human monocytes 1. Evidence for myeloperoxidase-linked and myeloperoxidase-independent candidacidal mechanisms. Journal of Clinical Investigation 55 : 338 - 46.

LEHRER,R.I. ; MITCHELL,K. ; HAKE,R. 1973 : Identification of a second fungicidal mechanism in the human neutrophil. Clinical Research 21 : 605.

LEIN,D.H. ; NIELSEN,S.W. 1975 : Experimental pathology of male bovine genital mycoplasmosis. Laboratory Investigations 32 : 450 - 1.

LIVINGSTON,C.W. ; GAUER,B.B. 1974 : Serological typing of T strain mycoplasmas isolated from the respiratory and reproductive tracts of cattle in the United States. American Journal of Veterinary Research 35 : 1467 - 71.

LOWELL PARSONS,C. ; GREENSPAN,C. ; MULHOLLAND,S.G. 1975 : The primary defence mechanism of the bladder. Investigative Urology 13 : 72 - 7.

LOWRY,O.H. ; ROSEBROUGH,N.J. ; FARR,A.L. ; RANDALL,R.J. 1951 : Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193 : 265 - 75.

LUKASHEV,I.J. ; ROTOV,V.I. ; ROZHNYATOVSKAYA,O.I. 1962 : Detection of the mycobacteria of paratuberculosis in bull semen. Veterinariya 39 : 28 - 30 . In Russian. Biological Abstracts 42 (1963) abstract 7132.

LUSIS,P.I. ; SOLTYS,M.A. 1971 : Pseudomonas aeruginosa. Veterinary Bulletin 41 : 169 - 77.

McARDLE,B. 1955 : A modified method for the microdetermination of citric acid. Biochemical Journal 60 : 647 - 9.

McFERRAN,J.B. 1962a : Poliovirus neutralising substances in cattle sera. Journal of Pathology and Bacteriology 83 : 73 - 83.

McFERRAN,J.B. 1962b : A substance in the sera of man and animals neutralising a bovine enterovirus. Journal of Pathology and Bacteriology 83 : 84 - 9.

MacMILLAN,W.G. ; HIBBITT,K.G. 1969 : The effect of antimicrobial proteins on the fine structure of Staphylococcus aureus. Journal of General Microbiology 56 : 373 - 7.

MacMILLAN,W.G. ; HIBBITT,K.G. 1973 : Surface properties and natural defence in mammals. Pesticide Research 4 : 863 - 70.

MANN,T.M. 1964 : " The Biochemistry of Semen and of the Male Reproductive Tract ". 2nd edition. Methuen & Co. Ltd., London,England.

MARDH,P.A. ; COLLEEN,S. 1974 : Lysozyme in the seminal fluid of healthy males and patients with prostatitis and in the tissues of the male urogenital tract. Scandinavian Journal of Urology and Nephrology 8 : 179 - 83.

MASON,D.Y. ; TAYLOR,C.R. 1975 : The distribution of muramidase (lysozyme) in human tissues. Journal of Clinical Pathology 28 : 124 - 32.

MATTHEWS,C.S. ; BUXTON,C.L. 1951 : The bacteriology of the cervix in cases of infertility. Fertility and Sterility 2 : 45 - 52.

MAULE,J.P. 1962 : The semen of animals and artificial insemination. Technical Communication 15. Commonwealth Agricultural Bureaux, Farnham Royal, Buckinghamshire, England. p 106.

MEREDITH,M.J. 1970 : Bacterial content of semen,collected by artificial vagina,from bulls that evert the preputial epithelium. Veterinary Record 87 : 122 - 4.

MIXNER,J.P. 1949 : Penicillin and sulfanilamide in semen diluters and their effect on the fertility of semen from relatively fertile bulls. Journal of Dairy Science 32 : 722.

MORRIS,C.J.O.R. ; MORRIS,P. 1964 : " Separation Methods in Biochemistry ". Pitman, London, England.

MOSAHEB,M.F. ; LADDS,A.H. ; LADDS,P.W. 1973 : The pathology of the external genitalia of bulls in Northern Australia. Australian Veterinary Journal 49 : 512 - 6.

MULLER-EBERHARD,H.J. ; NILLSON,U.R. ; DALMASSO,A.P. ; POLLEY,M.J. ; COLCOTT, M.A. 1966 : A molecular concept of immune cytolysis. Archives of Pathology 82 : 205 - 17.

NEW ZEALAND DAIRY BOARD. 1973/1974 : 50th Farm Production Report. The effect of Nystatin on Conception Rates.

NORMAN,C. ; McCOY,J.L. ; WILSON,H.A. ; PORTERFIELD,I.D. ; GOLDBERG,E. 1962 : Elimination of Vibrio fetus from suspensions of bovine sperm stored at low temperatures. American Journal of Veterinary Research 23 : 1019 - 22.

ODEBERG,H. ; OLSSON,I. ; VENGE,P. 1975 : Cationic proteins of human granulocytes IV : Esterase activity. Laboratory Investigation 32 : 86 - 90.

OLDS,P. ; OLIVER,L. ; SEATH,D.M. 1951 : The effect of antibiotics on the fertility of bull semen and their relationship to the estrous cycle length of dairy cattle following artificial insemination. Journal of Dairy Science 34 : 966 - 70.

OLSSON,I. ; VENGE,P. 1974 : Cationic proteins of human granulocytes II. Separation of cationic proteins of the granules of leukemic myeloid cells. Blood 44 : 235 -46.

ONOVIRAN,O. ; TRUSCOTT,R.B. ; FISH,N.A. ; BARKER,C.A.V. ; RUHNKE,H.L. 1975 : The recovery of mycoplasmas from the genital tract of bulls in A.B. units in Ontario. Canadian Journal of Comparative Medicine 39 : 474 - 5.

ORAM,J.D. ; REITER,B. 1968 : Inhibition of bacteria by lactoferrin and other iron chelating agents. Biochimica et Biophysica Acta 170 : 351 - 65.

OSSERMAN,E.F. ; LAWLOR,D.P. 1966 : Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. Journal of Experimental Medicine 124 : 921 - 51.

OSSERMAN,E.F. ; CANFIELD,R.E. ; BEYCHOK,S. 1974 : " Lysozyme ". Academic Press, New York,U.S.A.

PADGETT,G.A. ; HIRSCH,J.G. 1967 : Lysozyme, its absence in tears and leukocytes of cattle. Australian Journal of Experimental Biology and Medical Science 45 : 569 - 70.

PARRY,R.M. ; CHANDAN,R.C. ; SHAHANI,K. 1969 : Isolation and characterisation of human milk lysozyme. Archives of Biochemistry and Biophysics 103 : 59 - 65.

PARSONSON,I.M. ; SNOWDON,W.A. 1974 : Ephemeral fever virus, Excretion in semen of infected bulls and attempts to infect cows by intrauterine inoculation of the virus. Australian Veterinary Journal 50 : 329 - 34.

PARSONSON,I.M. ; SNOWDON,W.A. 1975 : The effect of natural and artificial breeding using bulls infected with, or semen contaminated with infectious bovine rhinotracheitis virus. Australian Veterinary Journal 51 : 365 - 9.

PARSONSON,I.M. ; AL-AUBAIDI,J.M. ; McENTEE,K. 1974a : Mycoplasma bovigenitalium : Experimental induction of genital disease in bulls. Cornell Veterinarian 64 : 240 - 64.

PARSONSON,I.M. ; CLARK,B.L. ; DUFTY,J.H. 1974b : Pathogenesis of Trichomonas fetus infection in the bull. Australian Veterinary Journal 50 : 421 - 3.

PHILLIPS,D.M.P. 1971 : " Histones and Nucleohistones ". Plenum Press, London, England.

PHILPOTT,M. 1969 : Studies on vibriosis in an A.I. stud. Veterinary Record 85 : 405 - 8.

POUNDEN,W.D. ; FERGUSON,L.C. ; KNOOP,L.C. ; KRAUSS,W.E. 1947 : A further report on Staphylococcal abortions in a dairy herd. Journal of American Veterinary Medicine Association 111 : 376 - 8.

PRINCE,P.W. ; ALMQUIST,J.O. ; REID,J.J. 1949 : Bacteriological studies of bovine semen II: the incidence of specific types of bacteria and the relation to fertility. Journal of Dairy Science 32 : 849 - 55.

RAZIN,S. ; ROZANSKY,R. 1957 : The responsibility of spermine for the antibacterial action of human semen. Journal of Laboratory and Clinical Medicine 49 : 877 - 81.

RAZIN,S. ; ROZANSKY,R. 1959 : The mechanism of the antibacterial action of spermine. Archives of Biochemistry and Biophysics 81 : 36 - 54.

REDDY,B.J. ; KRISHNAMURTHY,P.S. ; VENKATASWAMI,V. 1971 : Bacterial flora of prepuce and the effect of intra-preputial treatment on the bacteriological quality of semen. Indian Veterinary Journal 48 : 722 - 7.

REID,M.S. ; BIELESKI,R.L. 1968 : A simple apparatus for vertical flat sheet polyacrylamide electrophoresis. Analytical Biochemistry 22 : 374 - 87.

RICHARD,J.L. ; FICHTNER,R.E. ; PIER,A.C. 1976 : Yeasts in bovine semen. Cornell Veterinarian 66 : 362 - 8.

RICHARDSON,A. ; SMITH,P.J. 1968 : Herd fertility and Corynebacterium haemolyticum in bovine semen. Veterinary Record 83 : 156 - 7.

ROB,O. ; TOMAN,J. 1971 : Prevalence of fungi in the semen and prepuce of breeding bulls. Vet. Med. Praha 15 : 703 - 8. In Czech. Veterinary Bulletin 41 : abstract 2870.

ROZANSKY,R. ; GUREVITCH,J. ; BRZEZINSKY,A. ; ECKERLING,B. 1949 :Inhibition of the growth of Staphylococcus aureus by human semen. Journal of Laboratory and Clinical Medicine 34 : 1526 - 9.

ROZANSKY,R. ; BACHRACH,U. ; GROSSOWICZ,N. 1954 : Studies on the antibacterial action of spermine. Journal of General Microbiology 10 : 11 - 6.

ROZANSKY,R. ; RAZIN,S. ; GROSSOWICZ,N. 1954 : The antibacterial action of spermidine. Bulletin of the Research Council of Israel 4 : 306 - 7.

RUCKERBAUER,G.M. ; MALKIN,K. ; MITCHELL,D. ; BOULANGER,P. 1974 : Vibriosis, demonstration of Vibrio fetus and V. bubulus organisms in preputial fluid by immunofluorescence and cultural methods. Canadian Journal of Comparative Medicine 38 : 321 - 7.

RUEBKE,H.J. 1951 : Bacterial flora of the bovine male genitalia. American Journal of Veterinary Research 12 : 14 - 9.

SALISBURY,G.W. 1968 : Fertilising ability and biological aspects of sperm storage in vitro. 6th International Congress of Animal Reproduction and Artificial Insemination. Paris. Volume 2 : 1186 - 1204.

SALISBURY,G.W. ; BRATTON,R.W. 1948 : The fertility level of bull semen diluted at 1 : 400 with and without sulfanilamide. Journal of Dairy Science 31 : 817 - 22.

SALISBURY,G.W. ; FLERCHINGER,F.H. 1961 : In vitro ageing of sperm and evidence for embryonic or early foetal mortality in cattle. 4th International Congress of Animal Reproduction,Proceedings : 601 - 6.

SALISBURY,G.W. ; KNUDT,C.B. 1947 : The effect of sulfanilamide in the diluent upon fertility of bull semen. Journal of Dairy Science 30 : 361 - 9.

SALISBURY,G.W. ; WILLETT,E.L. ; GUNSALUS,I.C. 1939 : Some problems in bull semen storage. American Society of Animal Production,Proceedings 32 : 210 - 2.

SALISBURY,G.W. ; BRATTON,R.W. ; FOOTE,R.H. 1952 : The effect of time and other factors on the non-return to service estimate of fertility level in artificial insemination of cattle. Journal of Dairy Science 35 : 256 - 60.

SCHELLPFEFFER,D.A. ; HUNTER,A.G. 1970 : Electrophoretic and gel filtration behaviour of boar seminal plasma proteins before and after removal of the accessory sex glands. Journal of Reproduction and Fertility 23 : 291 - 8.

SCOTT FOSTER,J.H. ; RUSSELL,A.D. 1971 : Antibacterial dyes and nitrofurans. Chapter 3F. "Inhibition and Destruction of the Microbial Cell" edited by W.B. Hugo . Academic Press, London & New York.

SELLERS,R.F. ; BURROWS,R. ; MANN,J.A. ; DAWE,P. 1968 : Recovery of virus from bulls infected with foot-and-mouth disease. Veterinary Record 83 : 303.

SHANNON,P. 1973 : Factors affecting storage of semen. New Zealand Society of Animal Production, Proceedings 33 : 40 - 8.

SHANNON,P. ; TWOMEY,A. ; CURSON,B. 1974 : Antibacterial and anti-sperm effect of seminal plasma. New Zealand Society of Animal Production, Proceedings 34 : 61 - 4.

SHANNON,P. ; CURSON,B. ; TWOMEY,A. ; MOLAN,P.C. 1975 : Antibacterial activity of seminal plasma and other tissues. New Zealand Society of Animal Production,Proceedings 35 : 61 - 4.

SHEPARD,M.C. ; LUNCEFORD,C.D. 1970 : A urease colour test medium U_9 for the detection and identification of " T " mycoplasmas in clinical material. Applied Microbiology 20 : 539 - 43.

SINGLETON,E.F. ; SIMMONS,G.C. 1969 : Bacteriological study of semen from intensively housed bulls. Queensland Journal of Agricultural and Animal Sciences 26 : 1 - 7 .

SKARNES,R.C. ; WATSON,D.W. 1957 : Antimicrobial factors of normal tissues and fluids. Bacteriological Reviews 21 : 273 - 94.

SKERMAN,V.B.D. 1967 :"A Guide to the Identification of the Genera of Bacteria ". 2nd edition. Williams & Wilkins Co., Baltimore,U.S.A.

SLEIGHT,S.D. 1965 : The role of penicillin and streptomycin in the prevention of transmission of bovine leptospiras by artificial insemination. American Journal of Veterinary Research 26 : 365 - 8.

SMITH,H.A. ; JONES,T.C. ; HUNT,R.D. 1972 : " Veterinary Pathology ". Lea & Febiger, Philadelphia,U.S.A.

SOBER,H.A. 1970 : " CRC Handbook of Biochemistry. Selected Data for Molecular Biology ". editor. 2nd edition. The Chemical Rubber Co., 18901 Cranwood Parkway, Cleveland, Ohio, U.S.A.

SOBER,H.A. ; PETERSON,E.A. 1958 : Protein chromatography on ion exchange cellulose. Federation Proceedings 17 : 1116 - 26.

SOREL,M.P. 1961 : Contribution a l'etude de la bacteriologie du sperme frais de taureau. Academie Veterinaire de France, Bulletin 34 : 295 - 300.

SPRADBROW,P.B. 1968 : The isolation of infectious bovine rhinotracheitis virus from bovine semen. Australian Veterinary Journal 44 : 410 - 2.

STABLEFORTH,A.W. ; GALLOWAY,I.A. 1959 : " Infectious Diseases of Animals : Diseases due to Bacteria ". Butterworth Scientific Publications, London, England.

STAMEY,T.A. ; FAIR,W.R. ; TIMOTHY,M.M. ; CHUNG,H.K. 1968 : The antibacterial nature of prostatic fluid. Nature (London) 218 : 444 - 7.

SUBCOMMITTEE ON STAPHYLOCOCCUS AND MICROCOCCUS 1965 : International Bulletin on Bacterial Nomenclature and Taxonomy 15 : 109.

SULLIVAN,J.J. ; ELLIOTT,F.I. ; BARTLETT,D.E. ; MURPHY,D.M. ; KUZDAS,C.D. 1966 : Further studies on use of polymyxin B sulphate with dihydrostreptomycin and penicillin for control of Vibrio fetus in a frozen semen process. Journal of Dairy Science 49 : 1569 - 71.

SUNAIKIN,A.A. 1971 : Treatment of semen contaminated with Trichomonas fetus. Trudy vses. Inst. eksp. Vet. 37 : 184 - 9. In Russian. Veterinary Bulletin 41 : abstract 3407.

TALLGREN,L.G. ; RUSK,J. ; ALFTHAN,O.S. ; JOHANSSON,C-J. 1968 : Antibacterial activity in semen and its relation to semen quality. Annales Chiruvgiae et Gynaecologiae Fenniae 57 : 540 - 3.

TAYLOR,P.W. ; MORGAN,H.R. 1952 : Antibacterial substances in human semen and prostatic fluid. Surgery, Gynecology and Obstetrics 94 : 662 - 8.

TATLOR-ROBINSON,D. ; THOMAS,M. ; DAWSON,P.L. 1969 : The isolation of T mycoplasmas from the urogenital tract of bulls. Journal of Medical Microbiology 2 : 527 - 33.

TEW,J.G. ; SCOTT,R.L. ; DONALDSON,D.M. 1971 : Plasma Beta-lysin and lsyozyme following endotoxin administration and the generalised Schwartzman Reaction. Proceedings of the Society of Experimental Biology and Medicine 136 : 473 - 8.

TUNKL,B. ; ALERAJ,Z. 1965 : Discovery of the cause of paratuberculosis in bull semen from one station for artificial insemination. Jugoslovenski Veterinarski Glasnik 19 : 845 - 9. Biological Abstracts 49 (1968) : abstract 63372.

VAN TONDER,E.M. ; BOLTON,T.F.W. 1970 : The isolation of Actinobacillus seminis from bovine semen : a preliminary report. Journal of South African Veterinary Medicine Association 41 : 287 - 8.

VOGEL,A.I. 1951 : " A Textbook of Quantitative Inorganic Analysis, theory and practice ". 2nd edition. p 249. Longmans,Green & Co., London, England.

VON ULBRICH,F. ; HAASE,H. 1974 : Virus of IBR/IPV latent infection of bulls II. Identification of virus from pelletised bull semen. Monatshefte fur Veterinarmedizin 29 : 459 - 62.

WARDLAW,A.C. ; PILLEMER,L. 1956 : The bacteriocidal activity of the properdin system. Journal of Experimental Medicine 103 : 553 - 75.

WEBER,K. ; PRINGLE,J.R. ; OSBORN,M. 1972 : Measurement of molecular weights by electrophoresis on SDS acrylamide gel. In, " Methods in Enzymology". editors-in-chief = S.P.Colowick & N.O. Kaplan. Volume XXVI : Enzyme structure,part C. Edited by C.H.W. Hirs & S.N. Timasheff. Academic Press, New York & London.

WEBSTER,W.M. 1932 : Bovine sterility in New Zealand. Australian Veterinary Journal 8 ; 199 - 222 .

WHITE,M.B. ; SNOWDON,W.A. 1973 : Breeding record of cows inseminated with a batch of semen contaminated with Infectious Bovine Rhinotracheitis virus. Australian Veterinary Journal 49 : 501 - 6.

WILLETT,E.L. ; OHMS,J.L. 1955 : Field trials with semen containing several combinations of antibacterial agents. Journal of Dairy Science 38 : 1360 - 8.

WILLETT,E.L. ; OHMS,J.I ; FRANK,A.H. ; BRYNER,J.H. ; BARTLETT,D.E. 1955 : Nonreturn rate and embryonic mortality from inseminations by bulls with Vibrio fetus. Journal of Dairy Science 38 : 1369 - 74.

WILLIAMS,S.T. ; CROSS,T. 1971 : Isolation,purification,cultivation and preservation of Actinomycetes. In " Methods in Microbiology ",volume 4, edited by C. Booth. Academic Press, London & New York.

WILSON,A.T. ; ROSENBLUM,H. 1952 : The antistreptococcal properties of milk 1. Some characteristics of the activity of lactenin in vitro. The effect of lactenin on haemolytic streptococci of several serological groups. Journal of Experimental Medicine 45 : 25 - 38.

WINTER,A.J. ; BURDA,K. ; DUNN,H.O. 1965 : The evaluation of cultural techniques for the detection of Vibrio fetus in bovine semen. Cornell Veterinarian 55 : 431 - 44.

WINTER,A.J. ; SAMUELSON,J.D. ; ELKANA,M. 1970 : A comparison of immuno-fluorescent and cultural techniques for the demonstration of Vibrio fetus. Journal of American Veterinary Medicine Association 150 : 499 - 502.

YILMAZ,S. 1971 : The first isolation of Miyagawanella bovis from the semen of a bull. Etlik Veteriner Bakt,Enst. Dergisi (Turkey) 3 : 53 -73. In Turkish. Veterinary Bulletin 41 : abstract 5218.

YOUMANS,G.P. ; LIEBLING,J. ; LYMAN,R.Y. 1938 : The bacteriocidal action of prostatic fluid in dogs. Journal of Infectious Diseases 63 : 117 - 21.

ZEMJANIS,R. 1962 : " Diagnostic and Therapeutic Techniques in Animal Production :. Bailliere,Tindall and Cox Ltd., London, England.

ZGORNIAK-NOWPSIELSKA,I. ; BRANNY,J. 1970 : Mycoplasmas in the semen of bulls 1. Isolation and some properties of mycoplasmas from bovine semen. Medycyna Weterynarvina 26 : 51 - 3. In Polish. Veterinary Bulletin 40 (1971) : abstract 5510.

ZEYA,H.I. ; SPITZNAGEL,J.K. 1966a : Cationic proteins of polymorphonuclear leukocyte lysosomes 1. Resolution of antibacterial and enzymatic activities. Journal of Bacteriology 91 : 750 - 4.

ZEYA,H.I. ; SPITZNAGEL,J.K. 1966b : Cationic proteins of polymorphonuclear leukocyte lysosomes 2. Composition, properties and mechanism of antibacterial action. Journal of Bacteriology 91 : 755 - 62.

ZEYA,H.I. ; SPITZNAGEL,J.K. 1968 : Arginine-rich proteins of polymorpho-nuclear leukocyte lysosomes. Antimicrobial specificity and biochemical heterogeniety. Journal of Experimental Medicine 127 : 927 - 41.