THE ISOLATION AND CHARACTERIZATION OF ANTIBACTERIAL FACTORS FROM BOVINE SEMINAL PLASMA AND PANCREAS

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ABSTRACT

An isolation procedure was developed for the extraction of antibacterial factor(s) from bovine seminal plasma (BSP). The procedure involved a batch separation on a cation exchange resin: adsorption onto cellulose phosphate, and elution of the active fraction with sodium citrate. For comparison, an antibacterial fraction was extracted from bovine pancreas by homogenization of the tissue in an acidic-citrate solution, followed by treatment with cellulose phosphate. Optimal conditions for the recovery of antibacterial material with high specific activity were determined. The antibacterial fractions were further purified by ethanol precipitation and the purity of various preparations was monitored by SDS gel electrophoresis.

Purified bovine seminal plasma and pancreas preparations were used to immunize rabbits. The antisera obtained were run against homologous and heterologous bovine antigens in immunodiffusion agar plates. Whereas BSP preparations elicited a multicomponent antibody response in rabbits, pancreas preparations were either non-antigenic or only poorly antigenic.

Cellulose phosphate column chromatography of BSP ethanol precipitated material confirmed the heterologous nature of "purified" preparations. Absorption studies on immunodiffusion agar using anti-BSP antiserum and "column-antigens" were used to determine relationships between the multicomponent BSP antibody system (anti-BSP antiserum) and the various column fractions. Antibacterial activity was associated with one peak, which elicited an indistinct antibody response, compared to other components, which gave sharp precipitin

reactions in the presence of homologous antibody on immunodiffusion agar.

Physico-chemical and biological properties of the seminal plasma and pancreas preparations were established and compared. Antibacterial activity was determined by the agar diffusion assay using Micrococcus lysodeikticus as the test organism. The active fractions from both seminal plasma and pancreas material contained basic polypeptides with molecular weights of approximately 15,000 daltons. Both were dialyzable, heat stable, trypsin sensitive and were subject to little if any inactivation by anionic polymers such as deoxyribonucleic acid. Neither was associated with lysozyme or phospholipase activity.

An amino acid analysis of a seminal plasma preparation with a high specific activity gave a lysine to arginine ratio of 1.27; this is similar but not identical to the various calf thymus histone fractions which have been reported in the literature.

Further studies on BSP and pancreas material involved comparisons of ethanol and ammonium sulphate precipitations, as well as ethanol reprecipitation of BSP material following acidification in the presence of citrate. Although the active fractions of both seminal plasma and pancreas possessed similar characteristics, the different behaviour of these fractions in precipitation studies suggested that these may be closely related proteins or modified forms of the same protein.

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INTRODUCTION

INTRODUCTION

Many natural defence mechanisms are involved in the protection of animal hosts against invading microorganisms. Since the turn of the century, many animal tissues have been shown to contain substances which inhibit microbial growth. Skarnes and Watson (1957) reviewed the work on antimicrobial agents from normal tissues and body fluids, in an attempt to reduce the confusion which has developed regarding their identities and their role in natural resistance to infection. Much of this confusion has arisen from insufficient characterization of the various factors, and many of the more recent findings may simply represent rediscoveries of agents which had previously been inadequately defined.

Classification of antimicrobial substances has frequently been based on the tissue or fluid source, antimicrobial spectrum, heat stability and chemical composition. Other criteria such as measurements of potency, optimum pH, effects of inhibitors, and ionic influences may be useful in characterizing these factors (Skarnes and Watson, 1957). The degree of purity of a particular agent is of special importance, as are well defined experimental procedures, in order that meaningful comparisons may be made between one antimicrobial substance and another.

1. Antimicrobial Substances

Many of the antimicrobial agents from tissues and body fluids have been identified as polyamines, basic proteins or basic peptides.

1.1 Spermine

Spermine, a polyamine widely distributed in animal tissues, has been reported to have antibacterial activity against a variety of

microorganisms *in vitro* (Hirsch and Dubos, 1952; Rozansky *et al.*, 1954). Spermine may act indirectly, being first oxidized to spermidine by an amine oxidase, as observed in studies on the tubercle bacillus (Hirsch, 1953), or it may act directly to inhibit the growth of staphylococcus (Grossowicz *et al.*, 1955). Spermine is, at least partly responsible for the antibacterial effect of human semen (Rozansky *et al.*, 1949; Gurevitch *et al.*, 1951; Razin and Rozansky, 1957). Hirsch (1960) viewed the antimicrobial role of spermine *in vivo* as speculative since its action may be influenced by other substances, and would therefore depend on the environment; it may also be firmly bound to tissues and therefore ineffective against microorganisms.

Since human prostatic fluid is extremely rich in spermine (Tabor and Tabor, 1964), Fair and Wehner (1971) studied the effect of spermine against a variety of microorganisms, in an effort to determine the role of spermine in the natural defence against urinary tract infections in the human male. They demonstrated that at the normal acid pH of the prostatic secretions, spermine would have little, if any, inhibitory effect against the majority of organisms normally responsible for urinary infections.

1.2 Lysozyme

Lysozyme, a basic protein (Salton, 1957), is one of the most highly purified antimicrobial proteins which has been studied. The role of lysozyme in resistance to bacterial infection has never been clearly established (Skarnes and Watson, 1957). Dubos (1945) suggested that potentially pathogenic bacteria may be prevented from establishing themselves in the host due to the presence of lysozyme, which is widely distributed in tissues. Amano and coworkers (1954) presented evidence that lysozyme acts synergistically with complement and specific antibody,

greatly accelerating the lysis of several Gram negative pathogenic bacteria. Hirsch (1960) also suggested that lysozyme may kill bacteria in *in vivo* situations, since it acts within the physiological pH and ionic concentration range.

1.3 Histones and Protamines

The antimicrobial activity of histones was demonstrated in the late nineteenth century and confirmed by Miller *et al.* (1942), Negroni and Fischer (1944), Weissman and Graf (1947) and Hirsch (1958). The histones are a family of small, basic proteins associated with DNA in the cell nucleus (Phillips, 1962 ; Busch, 1965; Elgin and Weintraub, 1975). They possess large amounts of the basic amino acids, lysine and arginine (Crampton *et al.*, 1955) and the proportion of basic amino acids in each of the histones is about 25% (Phillips, 1962).

The histones are among the most highly modified proteins; the modifications include acetylation, methylation and phosphorylation (Elgin and Weintraub, 1975). Due to this high degree of modification, the histones are particularly sensitive to the procedures employed for their isolation and purification. Consequently, a harsh extraction procedure could yield a histone fraction quite different from that occurring naturally in a tissue.

The histones have been fractionated and the various fractions have been characterized and sequenced (Crampton *et al.*, 1955; Johns *et al.*, 1960; Johns, 1964; Kincade and Cole, 1966; Mauritzen *et al.*, 1967; Starbuck *et al.*, 1968; Bustin and Cole, 1969; Evans *et al.*, 1970; Sugano *et al.*, 1972). The different groups of histones as defined by amino acid analysis have recently been reviewed by Elgin and Weintraub (1975) (Table XXXIII).

Whereas histones 2a, 2b, 3 and 4 are highly conserved proteins,

histone 1 is more divergent (Elgin and Weintraub, 1975). Bustin and Cole (1968) have shown, for example, that there are a number of different subfractions of histone 1 within the tissues of a single species, and further that significant variation exists from one species to another.

With regard to antibacterial activity, the histones are heat stable under acid conditions, sensitive to proteolytic enzymes such as pepsin, and unstable in neutral phosphate buffer (Hirsch, 1958). Conflicting reports on the dialyzability of the histones could be due to the variability of dialysis tubing (Phillips, 1962).

The bactericidal action of the histones is dependent on the ionic concentration, and the general composition of the medium; salt concentrations only slightly higher than physiological levels abolish their bactericidal action (Hirsch, 1958). Hirsch (1960) believes that this salt effect, coupled with the fact that histones exist in tissues bound to nucleic acids, make the role of histones as antibacterial agents in normal tissues unlikely.

The protamines, also found complexed with nucleic acids, are basic polypeptides containing large concentrations of arginine (Kossel, 1928). The antibacterial effects of protamines, _in vitro_, have been known for many years (Phillips, 1962).

1.4 Basic Tissue Polypeptides

Considerable information on the antimicrobial activities of cationic polypeptides, and the sources from which they may be isolated has been accumulated over the past thirty years.

1.4.1 Early Work

In attempting to isolate factors concerned in the natural resistance of experimental animals to infection with _Bacillus anthracis_,

Bloom and coworkers (1947) succeeded in extracting a nondialyzable, anthracidal substance from the thymus, pancreas and caecum of different species. The rigorous isolation procedure involved an initial four day extraction, followed by heat treatment of the extract at 90^{o} for five minutes. Various fractions were then obtained by acetone and ethanol precipitation followed by extensive dialysis of each fraction.

The factor from calf thymus was described as an acid and heat stable basic polypeptide containing about 30% lysine and 3.5% arginine, and with an isoelectric point of approximately 11.2.

A tissue polypeptide from hog thyroid was also isolated and shown to cause clumping and precipitation of Bacillus megatherium, Staphylococcus aureus, a beta haemolytic streptococcus, and Escherichia coli (Bloom and Blake, 1948). Antibacterial factors were also isolated from bovine, rat and rabbit spleens (Bloom and Prigmore, 1952; Bloom et al., 1953).

Weissman and Graf (1947) suggested that the peptide isolated by Bloom and associates (1947) was derived from tissue nucleoprotein as a result of acid hydrolysis and exposure to an elevated temperature. They compared the anthracidal behaviour of calf thymus histone in an attempt to relate it to the tissue extracts.

Skarnes and Watson (1956a) further characterized the basic thymus peptide and showed that it was a protein-nucleic acid complex as originally proposed by Weissman and Graf (1947). Amino acid analysis showed it to be identical to histone fraction A reported by Crampton and coworkers (1955). The polypeptide was found to be most active at an alkaline pH and apparently more active against Gram positive bacteria.

In 1954, Dubos and Hirsch extracted a mycobactericidal peptide from calf thymus, and in lower yields from calf spleen, sheep thymus, beef

lymph nodes and calf pancreas. The isolation involved a 2 to 4 day acid extraction, followed by an overnight fractionation in picric acid-sodium hydroxide, pH 7.0, at room temperature. The precipitate obtained was resuspended and fractionated in 3% hydrochloric acid in 95% ethanol overnight at room temperature. A final acetone precipitation yielded the antibacterial substance.

Dubos and Hirsch (1954) suggested, like Bloom and associates (1947), that perhaps the peptide and related compounds may be released as a result of the autolytic processes accompanying inflammation or necrosis, making microbial survival in the tissues more difficult.

The peptide isolated by Dubos and Hirsch (1954) was basic, contained large amounts of lysine and arginine and had an isoelectric point between 10.0 and 11.0. The peptide was sensitive to the proteolytic enzyme, trypsin, and it was stable to autoclaving at pH 7, but not under basic or acidic conditions. Unlike the factor isolated by Bloom and coworkers (1947) this substance was dialyzable, suggesting a lower molecular weight histone fraction. Skarnes and Watson (1956a) observed that its amino acid composition was similar to histone fractions B and C of Crampton et al. (1955).

1.4.2 Leukocytes

A number of partially characterized antibacterial substances have been extracted from whole polymorphonuclear leukocytes by various investigators (Skarnes and Watson, 1957). Leukins, basic antibacterial proteins isolated by Skarnes and Watson (1956b), were thought, however, to represent protamine or histone fractions of nucleoprotein origin. In 1960 Cohn and Hirsch, using cell fractionation methods, demonstrated leukocytic antibacterial activity (phagocytin) in association with specific granules of polymorphonuclear leukocytes. Because these granules

also contained hydrolytic enzymes such as lysozyme, the nature of the antibacterial substances could not be defined independently. The histochemical studies of Spitznagel and Chi (1963) indicated that these substances may be polycationic in nature.

Zeya and Spitznagel (1966a) fractionated the guinea pig PMN leukocyte granules directly by electrophoresis into several components, three of which were basic antibacterial proteins distinct from lysozyme. For characterization studies the basic protein fraction was obtained by acid extraction followed by ethanol precipitation.

Amino acid analysis of the protein fraction showed a preponderance of basic amino acids, especially arginine (16%), and a comparison showed the proteins to be markedly different from the nuclear histones. Zeya and Spitznagel (1966b) suggested that the antibacterial activity, which Hirsch (1956a, 1956b) ascribed to phagocytin, may be due to lysosomal cationic proteins.

These proteins showed antibacterial activity against both Gram negative and Gram positive bacteria (Zeya and Spitznagel, 1966b). The basic protein fraction suppressed oxygen uptake by the bacterial cells, and damaged their permeability barrier as shown by a rapid release of ultraviolet absorbing material from the cells.

Anionic substances, such as nucleic acids, blocked the antibacterial activity of the protein fraction. One of the most important features of tissue and synthetic basic polypeptides is their inactivation by anionic polymers (Bloom et al., 1951; Burger and Stahmann, 1952; Katchalski et al., 1953; Skarnes and Watson, 1956; Spitznagel, 1961) or certain inorganic anions (Hirsch, 1954b). That is, anionic polymers of bacterial and tissue origin may neutralize the basic proteins of PMN granules, making them ineffective against invading bacteria. The

resistance-lowering activity of mucin, a negatively charged substance, (McLeod, 1941) may be partly attributed to neutralization of the cationic proteins from PMN leukocytes (Zeya and Spitznagel, 1966b).

Although the antagonizing effect of anionic substances makes it difficult to evaluate the role of cationic proteins in vivo, Zeya and Spitznagel (1966b) suggested that their antibacterial activity, in conjunction with their capacity to produce inflammation and tissue injury, may play an important role in host defence.

1.4.3 Bovine Teat Canal

Microorganisms invading the mammary gland may be subject to natural defence mechanisms in the teat canal and in the mammary gland itself (Hibbitt, 1970). The protective role of the teat canal may be due to its function as a mechanical barrier as well as to the presence of cationic proteins in the teat canal keratin. A number of cationic proteins have been isolated from bovine teat canal keratin (Hibbitt and Cole, 1968; Hibbitt et al., 1969; Hibbitt, 1970) by acid extraction of the keratin. The proteins were dialyzed extensively, then chromatographed on a carboxymethyl cellulose column and after sufficient washing with acetate buffer, the cationic proteins were eluted with 0.2M hydrochloric acid. Electrophoresis resolved the isolated proteins into six principle bands at pH 3.0.

In vitro studies have shown that these cationic proteins inhibit the growth of mastitis strains of staphylococcus and streptococcus. The cationic proteins produced marked changes in morphology of staphylococcus, particularly in the cell wall and plasma membrane. Similar changes were also seen in staphylococci recovered from the teat canal of a healthy cow, and in the presence of calf thymus histone (MacMillan and Hibbitt, 1969).

The cationic proteins caused an increased permeability of the bacterial cell membrane, as demonstrated by leakage of isotopically labelled protoplasmic proteins from the cells (MacMillan and Hibbitt, 1969; Hibbitt and Benians, 1971). These changes were similar to those observed by others with synthetic and natural basic polypeptides (Katchalski et al., 1952, 1953; Newton, 1956).

In vivo experiments showed that when Streptococcus agalactiae was deposited in goat teat canals, only one-sixth of the mammary glands became infected, whereas two-thirds of the glands became infected when a smaller number of organisms was injected directly into the teat cisterns of the same goats (MacMillan and Hibbitt, 1969). Other in vivo experiments showed that staphylococci recovered from the teat canal, could bind more ^{131}I labelled bovine serum albumin than control organisms; the former carried adsorbed, positively charged cationic proteins (MacMillan and Hibbitt, 1973; Hibbitt and Benians, 1971). The interaction between the cationic proteins and the anionic sites of bacteria, leads to interference in the ion binding and ion exchange of the bacterial cell, resulting in impaired integrity and inhibition of growth (MacMillan and Hibbitt, 1973).

The antimicrobial activity of the cationic proteins was lost in the presence of anionic proteins or other polyanions such as deoxyribonucleic acid, due to competitive binding and removal of the cationic proteins (Hibbitt, 1970). Hibbitt (1970) suggested that whole teat canal keratin exhibited no antibacterial activity because the cationic proteins were bound to the negatively charged proteins and nucleic acids; in the living animal however, the proteins would be synthesized continuously and might be free to bind to other negatively charged materials including microorganisms.

1.4.4 Bovine Milk Cells

Many of the properties of the teat canal proteins have also been observed in protein fractions isolated from bovine milk cells (Hibbitt, 1970), although polyacrylamide gel electrophoresis studies at pH 3.0 showed that more components (at least nine) could be isolated from milk cells by a similar extraction procedure to that used for the teat canal keratin. Proteins from both sources had isoelectric points between pH 7 and 9. The milk cell proteins also exhibited antimicrobial activity and were shown by Fast Green dye-staining to bind to the surface of staphylococci. Concentrations as low as 1 $\mu g/cm^3$ produced 50% growth inhibition.

The cationic proteins did not include lysozyme (Hibbitt et al., 1971). In fact, Padgett and Hirsch (1967) did not detect lysozyme in tears, saliva, nasal exudates or peritoneal leukocytes from cattle. In contrast, low levels (10-13 $\mu g/100\ cm^3$ milk) of lysozyme were detected by Parry et al. (1964) and Chandan et al. (1968). However, no indication of the location of the enzyme, whether in the milk cells or the cell free supernatant, was given. In contrast, human milk contains large quantities of lysozyme (40 $mg/100\ cm^3$ milk) (Chandan et al., 1968), which may be of major importance for the infant's defence against infection (Reddy et al., 1977).

The bovine milk cell cationic proteins retained their antimicrobial activity after being heated to 70^o for 30 minutes at pH 7.0, but retained only 10% of their activity at 100^o. The loss of antimicrobial activity between 70^o and 100^o was not unexpected as several fractions were extracted, and each might be inactivated at a different temperature (Hibbitt et al., 1971).

A short article by Trow-Smith (1975) summarized Hibbitt's findings

on the non-specific general defence mechanism provided by the cationic proteins. He suggested that the degree to which a cow mobilises these proteins, may determine the differences in resistance of cows to diseases such as mastitis.

1.4.5 Cervical Mucus

Brownlie and Hibbitt (1972) suggested that antimicrobial proteins in cervical mucus may also provide an initial, non-specific line of defence for the uterus against invading pathogens. The proteins were isolated by extraction of the cervical mucus with 0.5M sodium chloride, followed by extensive dialysis of the supernatants and chromatographic separation on a carboxymethyl cellulose column. The anionic proteins were eluted with acetate buffer and the cationic proteins were eluted as a single peak with 0.2M hydrochloric acid.

Whereas the anionic proteins showed no antimicrobial activity, the cationic proteins inhibited the growth of Staphylococcus aureus S305 and Brucella abortus S19. The latter had an inhibitory effect on the growth of staphylococci, with concentrations of 10-15 $\mu g/cm^3$ producing 50% inhibition. Electrophoresis of the cationic protein peak showed it to be a heterogeneous mixture, which resolved into four components at pH 3.0. Most of the proteins had isoelectric points between pH 7.0 and 8.6.

On the other hand, Rozansky and associates (1962) found no antimicrobial substance other than lysozyme in human cervical mucus. In bovine cervical mucus no lysozyme was detected by Brownlie and Hibbitt (1972), although this did not confirm the reports of Gibbons (1959), who demonstrated weak lysozyme activity, equivalent to 0.05 - 0.1% of that of crystalline egg-white lysozyme.

1.4.6 Bovine Whey

Howard et al. (1975) reported the presence of a heat stable (56^{o} for 30 minutes), dialyzable fraction in normal bovine whey capable of killing several species of bovine mycoplasmas. On the basis of its heat stability and dialyzability, they distinguished it from other bovine antibacterial agents such as: i) lactenin (Wilson and Rosenblum, 1952), ii) the cationic protein fraction from bovine teat canal keratin (Hibbitt et al., 1969), iii) the basic fraction present in cervical mucus (Brownlie and Hibbitt, 1972) and iv) the iron-binding protein, lactoferrin (Oram & Reiter, 1968). The lack of further characterization, however, makes comparison with other antimicrobial substances difficult.

Individual animals varied, both with respect to the general potency of the whey and the activity against specific strains of mycoplasmas. Howard and coworkers (1975) concluded that this antimicrobial factor may be one of the animal's defence mechanisms against mycoplasmal infection, and variation in synthesis of this factor may contribute to animal variation in susceptibility to mycoplasmal infections.

1.4.7 Prostatic Fluid

Stamey et al. (1968) observed that prostatic fluid in the dog possessed antibacterial activity against Gram negative and Gram positive bacteria. The fraction responsible for the activity of prostatic fluid was dialyzable, insensitive to trypsin, inactivated by either blood serum or trypticase soy broth and heat stable. Although the pH was not specified, the material retained its activity following treatment at 100^{o} for 30 minutes. Purification by gel filtration chromatography (Sephadex G-10) produced a constant peak of prostatic antibacterial factor, designated PAF. The antibacterial factor was a low molecular weight, cationic substance unrelated to spermine or lysozyme.

A similar antibacterial activity in the prostatic secretion of human males was also noted by Stamey and coworkers (1968). Although the reason why urinary tract infections are approximately ten times more frequent in women than men has never been adequately explained, the potential role of the antibacterial substance as a principal defence mechanism has been suggested by Stamey et al. (1968).

Fair and coworkers (1973) further studied the antibacterial action of canine and human prostatic fluid in an effort to elucidate the role of the normal prostate in the prevention of urinary tract infection. They also showed that various media had an antagonistic effect on the antibacterial activity of PAF. They studied a spectrum of Gram negative and Gram positive bacteria from patients with urinary infections and reported that about 90% of the organisms responsible for urinary tract infections were sensitive to the antimicrobial agent.

1.4.8 Seminal Plasma

Mammalian seminal plasma is a fluid of considerable biochemical complexity, comprising the combined secretions of the male accessory glands of reproduction (Mann, 1964; White, 1977). Larson and Salisbury (1954) studied bull seminal plasma and detected the presence of at least eleven (three major) electrophoretically distinguishable proteins or classes of proteins.

Forrester et al. (1969) isolated a basic polymeric material from bovine seminal plasma by precipitation at 80 to 100% ammonium sulphate saturation at 0^{o}, and subsequent cation exchange chromatography on carboxymethyl Sephadex. The protein had cell agglutinating properties, described in relation to erythrocytes and some other cell types from several mammalian species. These properties were ionic strength dependent. The molecular weight of the substance was about 48,000 and

the amino acid analyses revealed a high proportion of basic residues and also of sulphur containing amino acids. The high molecular weight and the high proportion of sulphur containing amino acids make it unlikely that the basic material is related to the basic nuclear proteins or small molecular weight basic polypeptides of bovine somatic tissues.

Sheid and associates (1976) observed the lysis and hydrolysis of white blood cells by bovine seminal plasma. They characterized the cell lysing agent as a nondialyzable substance, inactivated by heating at 100^{o} for 15 minutes or by treatment with the proteolytic enzyme, pronase. They identified the agent as acrosomal hyaluronidase. The proteolytic activity following cell lysis was shown to be due to chymotrypsin-like enzymes in seminal plasma and/or leakage of acrosomal trypsin-like enzymes.

Moore and coworkers (1976) have described basic proteins present in boar seminal plasma with high haemagglutinating activity. They studied the effects of seminal plasma proteins on spermatozoa and showed that spermatozoa from intact boars were affected to a greater extent than those from boars without seminal vesicles, presumably because the former could bind cationic proteins from seminal plasma.

The antibacterial activity of bovine seminal plasma was reported by Shannon et al. (1974) and Schollum et al. (1977). Previously, Shannon (1973) described the effects of seminal plasma on sperm "livability", and suggested that the toxic effect was due to an antibacterial substance. Compounds, such as egg yolk, which protected sperm also reduced the antibacterial effect of seminal plasma (Shannon et al., 1974). The activity was precipitated over a wide range with acetone, and dialyzable at pH 3.0 but not pH 7.0. Consequently, the

material was described as being heterogeneous, existing in various forms, ranging from a monomer to large molecular weight polymers.

Fractionation of bovine seminal plasma by gel filtration chromatography on Sephadex at pH 1.7 resulted in active peaks with apparent molecular weights ranging from 3,000 to 20,000 daltons (Shannon et al., 1975). The activity associated with the various peaks was thought to be due to aggregated forms of a monomeric peptide. A more complete dissociation of the peptide from other proteins was obtained at pH 12.0, although activity was lost after prolonged subjection to this pH. Shannon et al. (1975) suggested that the association of the peptide with larger proteins may be advantageous to the antimicrobial system, since the larger complex would be less likely to be excreted, and would perhaps be less susceptible to inhibition by anionic substances. Preliminary characterization of the inhibitory material in BSP by Schollum and associates (1977) indicated that the material had a minimum molecular weight of less than 50,000 daltons, but it readily reformed into larger aggregates.

Shannon and coworkers (1975) also demonstrated antibacterial activity in cell free extracts of pancreas, spleen, liver and lungs, which had been dialyzed for 15 days against distilled water at pH 7.0 before fractionation. Dialyzates obtained at pH 3.0 from bovine kidney, saliva, intestinal mucosa, serum leukocytes and teat canal epithelium also showed bactericidal activity. Shannon and associates claimed that all dialyzates had a major component with the same electrophoretic mobility as the peptide isolated from seminal plasma. According to Shannon et al. (1975), the occurrence of this peptide in many tissues represents a primary, non-specific, antimicrobial defence system.

1.5 Basic Synthetic Polypeptides

Watson and Bloom (1952) presented evidence which suggested that the antimicrobial activity of the tissue peptide, which they isolated, resided in the lysine residues within the molecule. A comparison of the antibacterial activity of the natural peptide, with that of a synthetic lysine polypeptide, showed that polylysine had approximately four times greater activity on a weight basis.

Katchalski and associates (1952, 1953) also showed that the synthetic basic polypeptides, such as polylysine, resembled the protamines and histones, low molecular weight basic polypeptides, in their antibacterial properties. Lysine polypeptides caused agglutination and growth inhibition of bacteria (Burger and Stahmann, 1952). They also caused a marked reduction in tobacco mosaic virus infectivity (Stahmann et al., 1951; Burger and Stahmann, 1951) and combined readily with red blood cells (Burger and Stahmann, 1951).

Spitznagel (1961) reviewed the action of cationic polypeptides and suggested that they combine with anionic sites in cell surface layers over a pH range 5.6 to 7.0. He studied the effects on the morphology of bacterial cells, and showed that the anionic dye, Fast Green, stained bacteria following treatment with cationic polypeptides, and that this binding paralleled a loss of bacterial viability.

Antibacterial activity has been found in many other crude extracts made from tissues, such as platelets (Skarnes and Watson, 1957), liver (Lewis and Schwartz, 1949), spleen (Myrvik, 1956) and lymph nodes (Soltys, 1952), but the antimicrobial substances have been insufficiently purified or characterized, making comparisons difficult, and therefore, they have not been included in this discussion.

2. Role of Basic Polypeptides in Natural Resistance

Although the cationic polypeptides have been shown to possess marked antibacterial activity *in vitro*, their role in the resistance to infection *in vivo* is difficult to assess, due to the lack of knowledge about the environment of cells and tissues. However, the effectiveness of these antimicrobial substances *in vivo* has been indicated by various findings.

Bloom *et al.*(1947) showed that a partially purified cationic polypeptide extracted from calf thymus, protected mice against experimental infection with *Bacillus anthracis*. That is, whereas no control mice survived the infection, 48% of the treated mice survived. Rubini *et al.* (1951) demonstrated the inhibition of growth of influenza virus in embryonated chicken eggs, when lysine polypeptides were injected with or before the virus. The coating of *Escherichia coli* with cationic proteins was observed by Spitznagel and Chi (1963) in histological sections from experimental skin infections. The *in vivo* experiments of MacMillan and Hibbitt (1969) and Hibbitt and Benians (1971) reported previously, demonstrated the association of cationic polypeptides with bacteria in the teat canal. Finally, Stamey *et al.* (1968) found that men with recurrent urinogenital infections lack the antibacterial peptide normally present in prostatic fluid. Such observations provide support for the *in vivo* involvement of cationic proteins in host resistance.

Two lines of thought have developed as to the occurrence and subsequent effectiveness of antimicrobial substances in normal tissues. Firstly, Skarnes and Watson (1957) believe that antimicrobial substances in tissues arise only in response to physiological changes which accompany stress. Thus, inflamed tissues may provide an excellent

environment for the liberation of reserves of these antimicrobial agents - basic peptides, amines and histones - not available for antimicrobial action in healthy tissue (Hirsch, 1960). On the other hand, antimicrobial polypeptides have been demonstrated in normal tissues (Shannon et al., 1975); Shannon and associates (1975) believe that basic peptides are important in the natural resistance of normal tissues, and that stress conditions provide a decrease in pH, which would cause greater dissociation of the peptide and therefore, increased antibacterial activity.

Often the preparative techniques used to isolate antimicrobial agents produce artifacts, which do not occur naturally in the tissues. However, the direct demonstration of antimicrobial activity in prostatic fluid and seminal plasma, for example, provides support for the view that an initial, non-specific defence mechanism exists which may kill or reduce the growth of invading microorganisms, thus allowing the more efficient operation of other host defences.

3. Aims of the Investigation

1. To isolate and purify antibacterial substances from bovine seminal plasma (BSP) and bovine pancreas. Gentle extraction procedures will be employed, in an effort to minimize modification of the antibacterial agents present in bovine seminal plasma and pancreas.

2. To characterize the antibacterial agent or agents in seminal plasma. The characterization to include studies on the nature of the antibacterial activity, amino acid analysis and molecular weight determination, as well as determination of

the effects of heat, proteolytic enzymes and anionic polymers on the antibacterial activity.

3. To compare and contrast the antibacterial substance from bovine pancreas with that from seminal plasma. The primary concern, with respect to this comparison, will be the production of antisera against both materials, followed by comparative immunodiffusion studies.