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.

MATERIALS

MATERIALS

1. Biological Materials

Bovine seminal plasma was obtained through the good offices of Mr. P. Shannon, Director of Research, N.Z. Dairy Board A.B. Centre, Hamilton; it was collected from vasectomized bulls, clarified by filtration and maintained at -20^{o} until used.

Human seminal plasma represented pooled samples that had been submitted to a clinical laboratory following vasectomies.

Whole pancreas from freshly killed bovines was obtained from the Longburn slaughter house. Each organ was placed in a plastic bag, frozen in dry ice, transported to the laboratory and maintained at -16^{o}.

Micrococcus lysodeikticus (2665), obtained from the National Collection of Type Cultures, was maintained on nutrient agar at 4^{o} after a 24 h incubation at 37^{o}.

2. Chemicals

Cellulose phosphate (capacity 0.97 - 0.99 meq/g, medium mesh) and deoxyribonucleic acid (type V, highly polymerized from calf thymus, and type III, highly polymerized from salmon) were obtained from Sigma. Bovine serum albumin (fraction V), cytochrome C (type II-A from horse heart), lysozyme (grade I, 3x crystallized from egg-white), ovalbumin (grade III), and trypsin (type III from bovine pancreas) were purchased from Sigma. β - lactoglobulin and α - lactalbumin were kindly donated by Dr. J. Tweedie, Department of Chemistry, Biochemistry and Biophysics,

Massey University; these had been prepared from bovine milk. Histone (types V-S (f1), VI-S (f2a), VII-S (f2b), and VIII-S (f3)) were isolated from calf thymus by Sigma by the method of Johns(1964). Sodium dodecyl sulphate (specially pure) was obtained from BDH Chemicals. All other chemicals were obtained from commercial sources and were of reagent grade. Deionized distilled water was used throughout the study. Dialysis tubing was purchased from H.B. Selby Co. and was soaked in deionized distilled water prior to use.

3. Media

3.1 Broth

Nutrient broth (Difco), nutrient broth + 0.3% yeast extract (BBL), trypticase soy broth (BBL), brain heart infusion broth (BBL), and antibiotic medium 3 (Difco) were prepared according to the manufacturers' specifications. All media were sterilized by autoclaving at 120^{o} for 15 min.

3.2 Agar

Solid media were obtained by adding 1.5% agar (Davis) to liquid media unless otherwise specified.

Citrated nutrient agar

nutrient broth	0.8 g
sodium citrate	0.66 g
agar	1.0 g
distilled water	100 cm^3

Blood agar

Noble agar (Difco) . . . . . 0.8 g

0.05M sodium acetate/sodium barbital

buffer, pH 7.5 . . . . . 100 cm^3

The media was cooled to 50^o and 5.0 cm^3 of blood was added; 20 cm^3 was used per Petri plate.

Egg Yolk agar

Noble agar . . . . . 0.8 g

0.05M sodium acetate/sodium barbital

buffer, pH 7.5 . . . . . 100 cm^3

The media was cooled to 50^o and 26.0 cm^3 was mixed with 0.25 or 0.5 cm^3 of egg yolk emulsion (Oxoid) and 0.25 cm^3 of 0.01M $CaCl_2$ and poured into a Petri plate.

Immunodiffusion agar

Noble agar . . . . . 1.3 g

sodium chloride . . . . . 8.0 g

distilled water to . . . . . 100 cm^3

Each Petri plate contained 20 cm^3 immunodiffusion agar.

4. Solutions

4.1 Protein Assays

4.1.1 Lowry Method

Reagent A : 1.0 cm^3 of 2.0% sodium tartrate was added to a mixture of 100 cm^3 of 2.0% Na_2CO_3 in 0.1M NaOH and 1.0 cm^3 of 1.0% $CuSO_4.5H_2O$.

Folin reagent : 2 volumes of distilled water were added to 1 volume of Folin reagent.

4.1.2 Protein - Dye Binding Method

Protein - dye binding reagent : 100 cm^3 of 85% (w/v) H_3PO_4 was added to 100 mg Coomassie Brilliant Blue G-250 dissolved in 50 cm^3 of 95% ethanol. Distilled water was added to a final volume of 1 litre.

4.2 Citric Acid Assay

Sulphuric acid - metaphosphoric acid solution (stable)

HPO_3	5.0 g
9N H_2SO_4	50 cm^3

Potassium permanganate - sodium bromide solution

5.0 g NaBr was added to 5.0 g $KMnO_4$ dissolved in 85 cm^3 of boiled, cooled distilled water. Distilled water was added to a final volume of 100 cm^3. The solution is stable for several weeks if stored in a brown stoppered bottle in the dark.

Thiourea solution, pH 9.2 (stable)

sodium borate	2.0 g
4% (w/v) thiourea solution . . .	100 cm^3

4.3 Gel Electrophoresis

SDS gel buffer, pH 8.9

Tris	12.1 g
glycine	7.5 g
SDS	1.0 g
distilled water to	1 litre

SDS denaturing buffer

urea	3.6 g
β- mercaptoethanol	0.1 cm^3
10% SDS	1.0 cm^3
SDS gel buffer to	10 cm^3

4.4 Sodium Citrate Solutions, pH 2.0

eg. 0.5M sodium citrate, pH 2.0

A solution of 14.7 g sodium citrate dissolved in 50 cm^3 of distilled water was adjusted to pH 2.0 with concentrated HCl and distilled water was added to a final volume of 100 cm^3.

METHODS

METHODS

1. Sample Analysis

1.1. Determination of Antibacterial Activity

1.1.1 By Agar Diffusion (Plate 1)

The antibacterial activity of a sample was determined using the agar diffusion method of Schollum et al. (1977). Micrococcus lysodeikticus (a 10 cm^3 nutrient broth culture incubated for 16 h at 37^o) was routinely used as the sensitive test organism. Turbidity of the bacterial suspension was determined against a broth blank in a Klett-Summerson colorimeter fitted with a blue filter (400 to 460 nm).

The bacterial suspension was adjusted to Klett 50 and 0.4 cm^3 was added to 16 cm^3 of citrated nutrient agar (Materials 3.2) at 50^o (about 8×10^5 colony forming units per plate). Wells, 9.5 mm in diameter, were cut in the solidified seeded agar with a metal bore, and the agar aspirated from each well. Each well was filled with 0.1 cm^3 of a sample using an autopipette fitted with a replaceable tip. The samples were allowed to prediffuse into the agar for 3 h at 4^o; the plates were then incubated for 24 h at 37^o.

Zones of inhibition were measured to the nearest 0.1 mm with Vernier calipers. All samples were assayed in triplicate. Samples yielding a zone diameter greater than 22.0 mm were reassayed using a higher sample dilution in distilled water. Although the samples tested were not sterile this did not present a major problem; only occasionally did contamination occur around a well.

The zone diameter was converted into arbitrary units /cm^3 based on lysozyme activity as a standard (Fig. 1; Schollum, 1977). In this

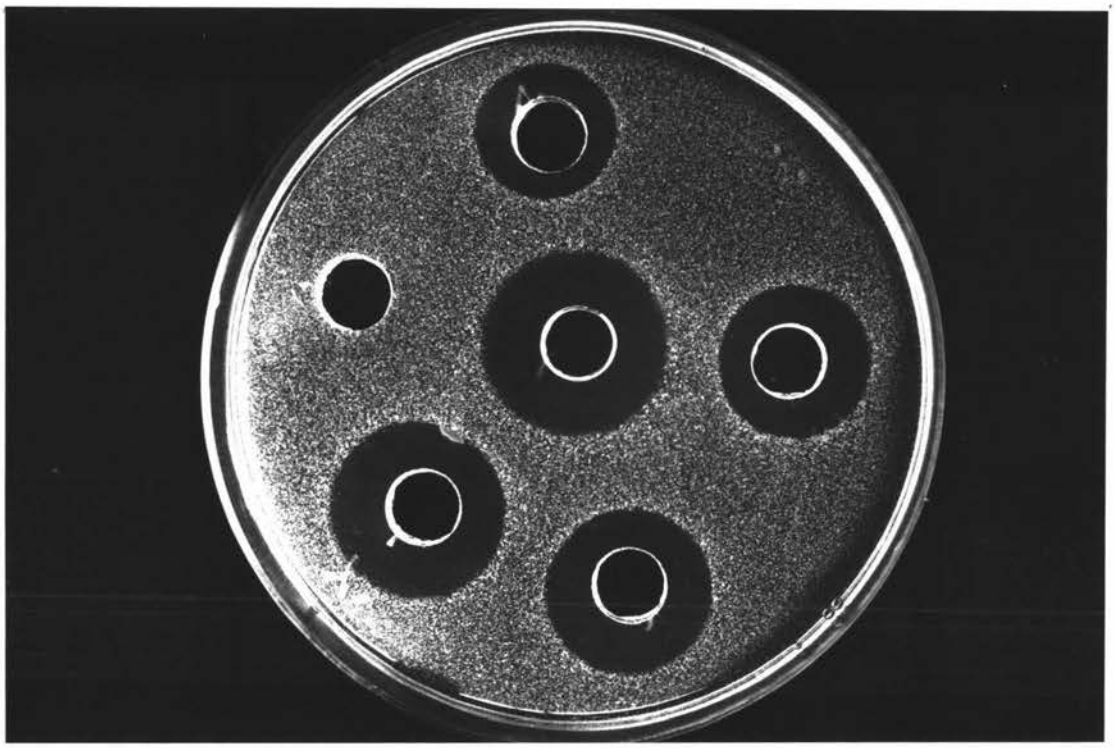


Plate 1: Determination of antibacterial activity by the agar diffusion assay. Note the zones of no-growth around 5 of the sample wells.

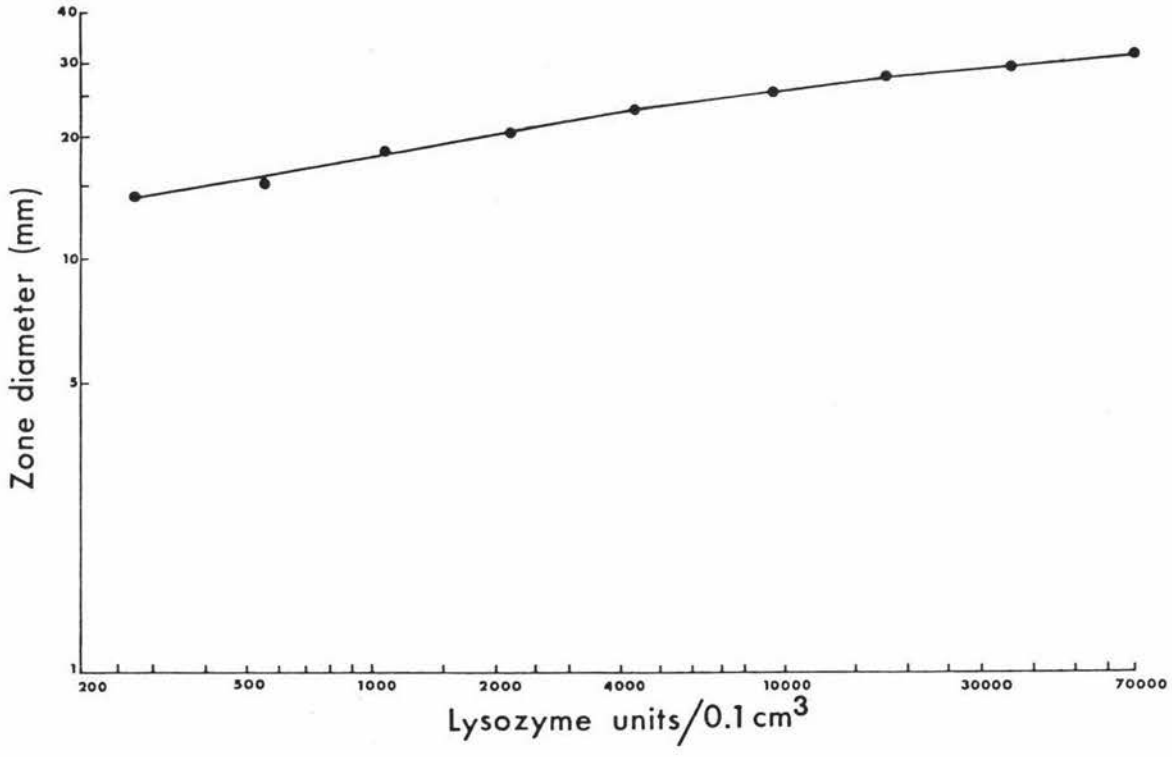


Fig. 1 Determination of antibacterial activity by the agar diffusion assay (Schollum et al., 1977).

system, samples producing no zone of inhibition were assumed to have less than 275 arbitrary units/cm^3, which represents the diameter of the well and consequently the lower limit of the assay. The lysozyme used for the standard curve had 35,000 units/mg; one unit of lysozyme activity is defined as the amount of enzyme which produces a 0.001/min decrease in absorbance at 450 nm, pH 6.24 and 25^o, in a 2.6 cm^3 suspension of M. lysodeikticus.

1.1.2 By Broth Dilution

Minimal inhibitory concentrations were determined by the broth dilution technique. An appropriate volume of broth was added to each of a series of sterile 13 x 100 mm tubes and a known volume of antibacterial substance was added to the first tube. Serial two-fold dilutions were performed aseptically through a series of 6 to 10 tubes; an additional tube was included as a control. A 1.0 cm^3 volume of each dilution was inoculated with 0.05 cm^3 from a M. lysodeikticus nutrient broth culture in the logarithmic phase of growth, to give 1 x 10^5 colony forming units/cm^3 of test dilution, as recommended by Ericsson and Sherris (1971). The tubes were mixed and incubated at 37^o for 24 h, read and reincubated for an additional 24 h, and then reread; this extended incubation was useful in determining doubtful positive tubes. Turbidity was noted in positive growth tubes after gentle mixing of the contents. The least amount of antibacterial substance which caused the complete inhibition of growth was recorded as the minimal inhibitory concentration (MIC).

The effect of different media on the MIC of a given BSP preparation was tested. The effect of pH on the MIC was determined by incorporating an appropriate buffer at 0.05M in the medium.

The test sample was sterilized either by autoclaving at 120° for 15 min, or by chloroform treatment. The latter involved the addition of 2 to 4 drops of chloroform to a sample in a 5 cm^3 screw cap bottle. The contents were mixed, allowed to stand for 5 to 10 min, and the dissolved chloroform removed by aerating the solution for 10 min with a sterile Pasteur pipette. Serial dilutions of a chloroform treated saline control indicated the method was sufficient to remove chloroform and allow growth of the test organism.

1.2 Determination of Protein

1.2.1 Lowry Method

Protein was routinely determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard (Fig. 2). The protein standard was prepared gravimetrically and adjusted to 200 $\mu g/cm^3$ such that the absorbance of the solution at 280 nm was 0.132 (Kirschenbaum, 1970). Five cm^3 of freshly prepared reagent A (Materials 4.1.1) was added to 1.0 cm^3 of sample containing 5 to 100 μg of protein. The mixture was vortexed and allowed to stand for 10 min at room temperature. Then 0.5 cm^3 of diluted Folin reagent was added, and the contents were mixed and allowed to stand for 30 min at room temperature. The absorbance at 660 nm was then determined on a Unicam SP 1800 spectrophotometer.

1.2.2 Protein -Dye Binding Method

For comparison, the protein - dye binding method of Bradford (1976) was used for several protein determinations of purified BSP and pancreas preparations using bovine serum albumin as a protein standard (Fig. 3). Five cm^3 of protein reagent (Materials 4.1.2) was added to a 0.1 cm^3 sample containing 10 to 100 μg of protein, and the contents mixed by

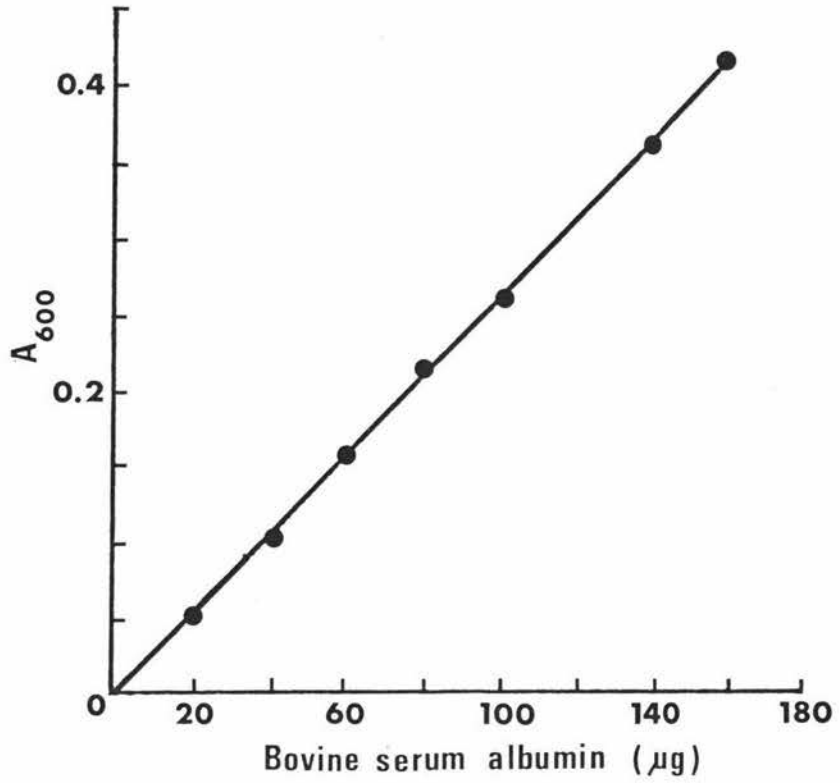


Fig. 2 Determination of protein by the Lowry method

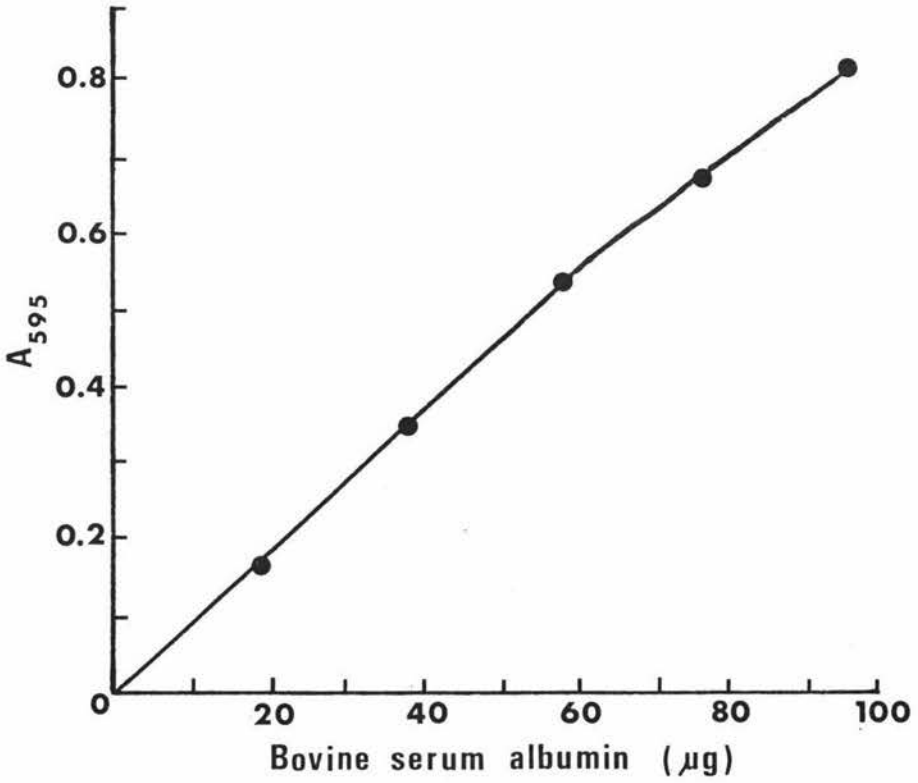


Fig. 3 Determination of protein by the protein-dye binding method

vortexing. The absorbance at 595 nm was measured after 2 min and before 1 h against a reagent blank.

1.3 Determination of Citric Acid

The amount of citrate present in various preparations was determined by the procedure described by Camp and Farmer (1967), based on the formation of pentabromoacetone from citric acid and subsequent color development with thiourea (Fig. 4). A standard citric acid solution was prepared by dissolving 0.1093 g of crystalline citric acid ($C_6H_8O_7.H_2O$) in 10 cm^3 of 9N H_2SO_4 and the volume adjusted to 100 cm^3 with distilled water. This solution contained 1.0 mg of anhydrous citric acid/cm^3, and 10 cm^3 of this standard solution diluted to 100 cm^3, gave a working citric acid standard of 100 $\mu g/cm^3$. Both solutions were stored at 4^o. The assay was as follows:

1. One cm^3 of sulphuric acid - metaphosphoric acid solution (Materials 4.2) was added to 1.0 cm^3 of protein-free sample or working standard in an acid washed, glass-stoppered test tube and placed in an ice bath. Deproteinization of samples was attempted by the precipitation of protein with 10% trichloroacetic acid (v/v), followed by centrifugation to yield a protein-free supernatant. However, due to the very low protein concentrations present following the dilution of a sample for citrate determination, no visible precipitate was formed and the deproteinization step was generally omitted.
2. While gently shaking the tube, 2.0 cm^3 of potassium permanganate - sodium bromide solution (Materials 4.2) was added and the tube was returned to the ice bath for 10 min.

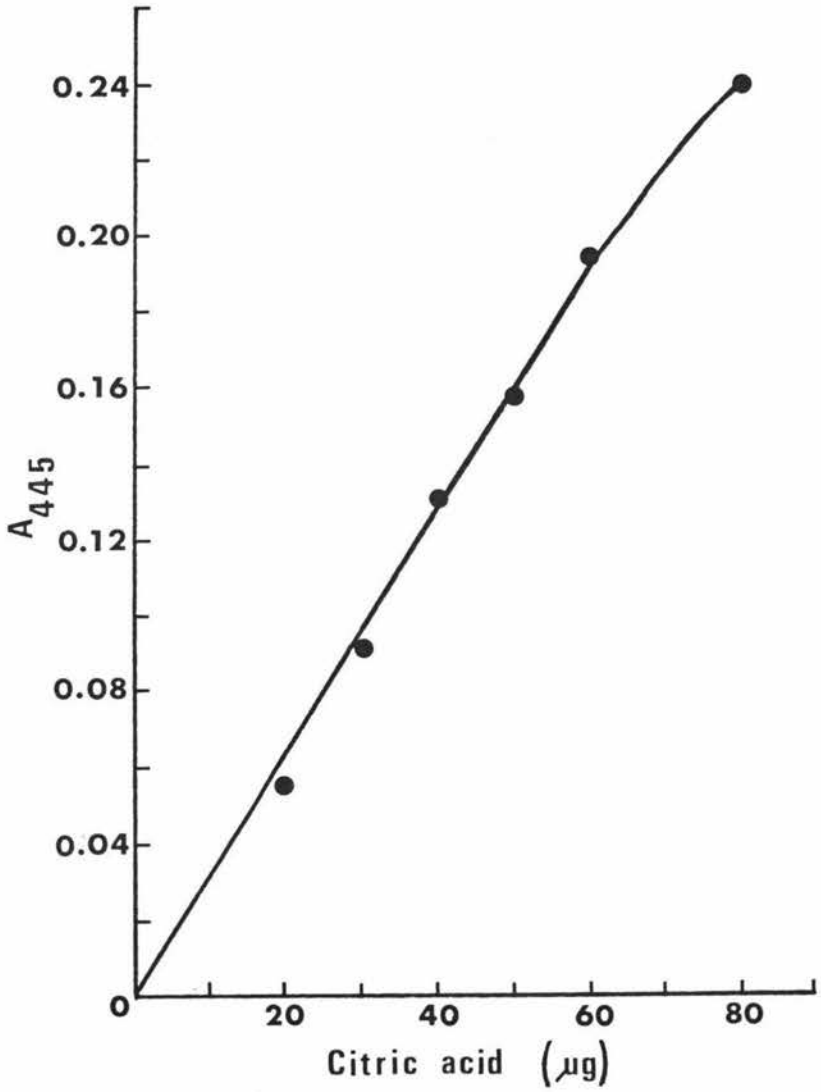


Fig. 4 Determination of citric acid

3. While vortexing the contents of the tube, 3.0% hydrogen peroxide (v/v) was added dropwise until the solution became colorless.
4. 1.5 cm^3 of petroleum spirit (b.p. 80-100^o) was added and the tube vortexed for 30 sec to extract the pentabromoacetone from the aqueous phase.
5. A 1.0 cm^3 aliquot of the non-aqueous layer was transferred to a glass-stoppered test tube and 3.5 cm^3 of thiourea solution (Materials 4.2) was added. The contents were mixed by vigorous vortexing for 1 min.
6. The absorbance at 445 nm of the aqueous layer was measured against a blank which was prepared by mixing 1.0 cm^3 of petroleum spirit with 3.5 cm^3 of thiourea solution. The standard curve was linear up to 70 μg citric acid/cm^3 (Fig. 4). Modifications in the procedure, which included the use of less than 1.0 cm^3 of the non-aqueous layer during color development, were helpful in obtaining a color intensity which fell within the linear range.

2. Isolation and Purification of Antibacterial Factors from Bovine Seminal Plasma and Pancreas

2.1 Extraction with Deoxyribonucleic Acid

The extraction of antibacterial material from bovine seminal plasma (BSP) using DNA was outlined by Schollum (1977) as follows:

1. To 0.15 cm^3 of 0.2M sodium phosphate buffer, pH 8.0, was added 1.0 cm^3 of bovine seminal plasma, which had been adjusted to pH 8.0 with 0.13M NaOH.

2. The buffered seminal plasma was transferred to 10 cm^3 graduated centrifuge tubes and 10 mg of DNA was added per cm^3 of buffered BSP.
3. The tubes were capped and heated in a 60^o bath for 1 h with occasional, gentle mixing by inversion.
4. The tubes were cooled on ice and 5.6 mg of NaCl was added per cm^3 of solution, giving a final concentration of 0.14M NaCl. Two controls, heated and unheated but without DNA, were carried to this stage and later assayed for antibacterial activity.
5. The precipitated protein-DNA complex was pelleted by centrifugation and the supernatants set aside to be tested for antibacterial activity.
6. The protein-DNA pellets were washed 3 times with 0.14M NaCl and the 3 washes were tested for antibacterial activity.
7. The protein-DNA complex was dissolved in chilled, 1.6M sodium citrate, and transferred to a beaker; distilled water was added to give a final concentration of 0.4M sodium citrate. The final volume of the solution was equivalent to the initial volume of buffered BSP used.
8. The DNA was precipitated from solution with an equal volume of absolute ethanol at room temperature; clumping of the DNA was facilitated by swirling the beaker. The DNA was removed by centrifugation and the supernatant stored at -20^o overnight.
9. The following day the solution was centrifuged at 12000 x g for 15 min in a Sorvall RC2-B centrifuge. The supernatant was collected and the precipitate, designated ppt. 1:1, was transferred to a small beaker with several cm^3 of distilled water.

10. A further 2 volumes of absolute ethanol were added to the supernatant to yield a second precipitate (1:3). The supernatant and precipitate were collected as described above.
11. Finally, an additional 4 volumes of absolute ethanol were added to the supernatant, and a third precipitate (1:7) and a final supernatant were collected by centrifugation.
12. The three precipitates and an aliquot of the final supernatant were lyophilized, resuspended in a known volume of distilled water, and assayed for antibacterial activity. Precipitates 1:1 and 1:3 were assayed at concentrations of 5 and 10 mg of precipitate/cm^3.

2.2 Preparation of Crude Pancreas Extract

Initial studies involved the homogenization of pancreas tissue in a variety of solutions. The following procedure gave extracts with maximum antibacterial activity.

Frozen pancreatic tissue was cut into 1 to 2cm pieces and the fat removed. Throughout the procedure the tissue was kept chilled to minimize the production of breakdown products by proteolytic enzymes. The tissue was weighed and homogenized in a chilled Waring blender in a volume of chilled, 0.2M sodium citrate, pH 2.0, equal to the weight of tissue. The tissue was blended for four, 5 sec intervals at high speed, two, 10 sec intervals at low speed, and finally for 10 sec at high speed with 5 sec intervals between each blending.

The homogenate was transferred to an Erlenmeyer flask as a shallow layer and heated with occasional swirling for 1 h in a 65^{o} bath. The flask was cooled on ice and the contents centrifuged at 12000 x g for 20 min at 0^{o}. The supernatant was collected and clarified of any solidified fat by filtration through muslin. The pH of the pancreas

extract was then adjusted to pH 7.4 with 6M NaOH.

2.3 Extraction with Cellulose Phosphate

2.3.1 Preparation of Cellulose Phosphate

Cellulose phosphate was equilibrated over several days as outlined by Miller (1973). The resin was suspended in distilled water and allowed to settle for 20 to 30min several times, and the fine particles in the supernatant were poured off after each settling. The resin was washed in 5 volumes of 0.5M NaOH for 15 to 20 min, and then neutralized to pH 8 by washing with water on a sintered glass funnel. The cellulose phosphate was then washed in 5 volumes of 0.5N HCl for 15 to 20 min and neutralized to pH 6 by washing with water. The resin was suspended in the desired buffer, 0.1M sodium phosphate buffer, pH 7.4, unless otherwise indicated, and titrated with 6M NaOH to the appropriate pH. After several hours the pH was checked and the resin was retitrated if necessary. The buffer was changed regularly until the pH and ionic strength were stabilized.

Ten g of cellulose phosphate was found to be a convenient amount for equilibration and yielded about 200 cm^3 of slurry. Routine correlation of the cellulose phosphate slurry volume with the wet weight of cellulose phosphate was obtained by centrifugation of several 1.0 cm^3 samples of slurry at 4000 rpm for 15 min in a bench-operated, Sorvall, angle head centrifuge (type A rotor). The supernatants were removed with a Pasteur pipette and each wet pellet was weighed. The slurry was adjusted so that 1.0 cm^3 of slurry gave a wet pellet weight of between 0.60 and 0.65g. For comparison, this corresponded to 0.40 to 0.43 g following centrifugation of 1.0 cm^3 of slurry at 4000 rpm for 15 min in a bench-operated, BTL, "swing-out" head centrifuge.

The slurry was stored at 4^o until ready to use.

2.3.2 Determination of Optimal Conditions for Extraction with Cellulose Phosphate

2.3.2.1 Bovine Seminal Plasma

2.3.2.1.1 Adsorption of the Antibacterial Factor(s) to Cellulose Phosphate (CP)

Initially an experiment was done to determine whether antibacterial factor(s) present in BSP could be adsorbed to cellulose phosphate and subsequently eluted. A mixture of 2.5 cm^3 of BSP and 3.0 cm^3 of cellulose phosphate slurry in 0.1M sodium phosphate buffer, pH 7.4, was prepared in a 10 cm^3 beaker and mixed by stirring with a glass rod at room temperature. At 5, 15, 30 and 60 min 1.0 cm^3 samples were removed and centrifuged at 4000 rpm for 15 min. The supernatants were collected and assayed for antibacterial activity. The pellets were resuspended in 0.5 cm^3 of 1.6M sodium citrate and allowed to stand for 15 min with occasional mixing. Then 2.0 cm^3 of distilled water was added, thereby reducing the citrate concentration to 0.32M, as citrate concentrations greater than 0.5M interfere in the determination of activity by the agar diffusion method. Each suspension was mixed, centrifuged and the final eluates were assayed for antibacterial activity. The activity of untreated BSP was also determined.

2.3.2.1.2 Effect of Varying the Relative Amount of Cellulose Phosphate

i. Various volumes of CP slurry were added to an appropriate volume of BSP to give ratios of 0.25, 0.5, 1.0, 2.0, and 3.0 cm^3 of CP slurry to 2.5 cm^3 of BSP. The procedure was as outlined in Methods 2.3.2.1.1, except that each CP pellet was washed once with 2.0 cm^3 of 0.1M sodium phosphate buffer, pH 7.4, before treatment with sodium citrate.

The washes were collected by centrifugation and assayed for antibacterial activity.

ii. The experiment described above was repeated using 0.25, 0.5, 1.0, 2.0 and 3.0 cm^3 of CP slurry and 2.5 cm^3 of BSP, and the final volume was adjusted to 5.5 cm^3 with 0.1M sodium phosphate buffer, pH 7.4. This experiment was repeated three times with variations in the kinds and volumes of washes used.

iii. A variation on the study of BSP - CP ratios was conducted on mixtures of 1.0 or 3.0 cm^3 of CP slurry and 2.5 cm^3 of BSP. The volumes were not adjusted with buffer and after 10 min the BSP - 3.0 cm^3 CP mixture was divided into 3 aliquots and the procedure was continued as outlined in Methods 2.3.2.1.6.ii.a., using a 0.5M NaCl wash. In this experiment then, the BSP - CP ratio was varied, but all washes were done on a constant volume of cellulose phosphate (1.0 cm^3).

iv. A final experiment, related to the adsorption of antibacterial activity to cellulose phosphate, was conducted by mixing 0.25 and 0.5 cm^3 of CP slurry with 2.5 cm^3 aliquots of BSP and the volume made to 3.0 cm^3 with buffer. The first supernatants were collected and 0.35 cm^3 of each was removed and tested for antibacterial activity. Buffer washes and final supernatants were also collected. The remainder of each first supernatant was treated with 3.0 cm^3 of CP slurry and the supernatants, washes and final supernatants were collected and assayed.

2.3.2.1.3 Effect of Heat on the Adsorption of the Antibacterial Factor(s) to Cellulose Phosphate

Two mixtures containing 1.5 cm^3 of CP slurry and 1.25 cm^3 of BSP were prepared. One mixture was left at room temperature for 1 h and the other was heated at 60^o for 1 h. First supernatants, buffer washes

and final supernatants were collected and assayed. The antibacterial activities of heated and unheated BSP controls were also determined.

2.3.2.1.4 Effect of Varying the pH of the Cellulose Phosphate Slurry

To determine the effect of pH on the adsorption of antibacterial material to cellulose phosphate, 2.5 cm^3 of BSP was mixed with 0.5 cm^3 of CP slurry equilibrated in 0.1M sodium phosphate buffer at pH 5.8, 7.4 and 8.0. The first supernatants were collected, and each CP pellet was washed with buffer of the same pH as that used initially. Buffer washes and final supernatants were collected and assayed.

In all succeeding batch treatments cellulose phosphate was equilibrated in 0.1M sodium phosphate, pH 7.4.

2.3.2.1.5 Acid - Citrate - Heat Treatment of BSP

A volume of BSP was treated with an equal volume of 0.2M sodium citrate, pH 2.0 and the solution was heated in a capped universal bottle for 1 h at 65^{o}. This treatment corresponded to that used during the preparation of pancreas extract.

The solution was centrifuged at 12000 x g for 15 min and the pH of the supernatant was adjusted to pH 7.4 with 6M NaOH. The supernatant was assayed for antibacterial activity. The treated supernatant (2.5 cm^3) was mixed with 0.5, 1.0, 2.0 and 3.0 cm^3 of CP slurry and the volume was adjusted to 5.5 cm^3 with buffer. The procedure was continued as outlined in Methods 2.3.3.2 for a small batch treatment, and first and final supernatants were collected and assayed.

2.3.2.1.6 Elution of Proteins Bound to Cellulose Phosphate

i. Elution with Sodium Citrate

Initially 0.5 cm^3 of 1.6M sodium citrate was used to elute

antibacterial activity from a 3.0 cm^3 CP pellet, and subsequently, the citrate was diluted five times with 2.0 cm^3 of distilled water before the final supernatant was collected by centrifugation. The results obtained were compared with those in which 1.0 cm^3 of saturated sodium citrate (22^o) was used and diluted after 15 min with 5.0 cm^3 of water. A third 3.0 cm^3 CP pellet was washed with 1.5 cm^3 of 1.6M sodium citrate diluted after 15 min with 6.0 cm^3 of distilled water or 0.1M sodium phosphate buffer, pH 7.4

ii. Elution with Sodium Chloride

a. Variation of NaCl Concentration

The use of NaCl to remove contaminating proteins from cellulose phosphate treated with BSP was studied. In initial experiments, 4.0 cm^3 of BSP were added to 4.8 cm^3 of CP slurry (2.5 cm^3 BSP : 3.0 cm^3 CP) and after 10 min with occasional stirring the mixture was divided into 1.8 cm^3 aliquots in 13 x 100 mm tubes (equivalent to 1.0 cm^3 of CP slurry per tube). The first supernatants were obtained by centrifugation. Each pellet was washed with 2.0 cm^3 of buffer and the wash obtained by centrifugation. Each pellet was then washed with 2.5 cm^3 of one of the following: 0.25, 0.75 or 1.0M NaCl; the charged resin was held in the wash solution for 15 min with occasional mixing and the NaCl washes were obtained by centrifugation. The pellets were washed again with buffer and then with 0.5 cm^3 of 1.6M sodium citrate to yield a second buffer wash and final supernatant. Each fraction was assayed. A similar experiment compared the effects of 0.25, 0.4, 0.6 and 0.75M NaCl washes.

b. Variation of NaCl Volume

Another experiment (2.5 cm^3 of BSP + 3.0 cm^3 of CP slurry) compared the use of a single 7.5 cm^3 0.5M NaCl wash with that of three, 2.5 cm^3 washes for the removal of protein and activity from cellulose

phosphate. This experiment was repeated using 0.2M NaCl. A final experiment compared the use of one or two, 7.5 cm^3 0.2M NaCl washes with that of six, 2.5 cm^3 washes for the elution of contaminating proteins from cellulose phosphate. The latter experiment also compared the use of a 15 min final wash of 1.5 cm^3 of 1.6M sodium citrate, subsequently diluted with 6.0 cm^3 of water, with that of a 7.5 cm^3 wash of 1.0M NaCl for the elution of antibacterial material from cellulose phosphate.

iii. Elution with Sodium Phosphate

Various concentrations of sodium phosphate buffer, pH 7.8, were used in an attempt to elute antibacterial activity adsorbed to CP. For all reactions 2.5 cm^3 of BSP was added to 3.0 cm^3 of CP slurry in 0.1M phosphate buffer, pH 7.4 and mixed occasionally for 10 min. The first supernatants were obtained by centrifugation and the pellets were washed with 2.0 cm^3 of 0.5M sodium phosphate, pH 7.8. The buffer washes were obtained by centrifugation and the pellets were washed with 1.5 cm^3 of one of the following: 0.1, 0.25, 0.4 or 0.5M phosphate buffer, pH 7.8. These washes were subsequently diluted with 6.0 cm^3 of distilled water. The ionic strength (μ) for these buffers is 0.6, 1.5, 2.4 and 3.0 respectively. The final supernatants were obtained by centrifugation and were compared with those obtained by washing the pellet with 1.0M NaCl (μ = 1.0) or 1.6M sodium citrate (μ = 9.6).

2.3.2.2 Pancreas Extract

2.3.2.2.1 Adsorption of the Antibacterial Factor(s) to Cellulose Phosphate

An initial experiment, as described in Methods 2.3.2.1.1, was

conducted on 2.5 cm^3 of crude pancreas extract.

2.3.2.2.2 Effect of Varying the Relative Amount of Cellulose Phosphate

Various volumes of CP slurry, 0.5, 1.0, 2.0 and 3.0 cm^3, were added to 2.5 cm^3 of pancreas extract and the volume was adjusted to 5.5 cm^3 with 0.1M phosphate buffer, pH 7.4. The procedure was continued as outlined in Methods 2.3.3.2 and the various washes were assayed.

2.3.2.3 Human Seminal Plasma (HSP)

2.3.2.3.1 Adsorption of the Antibacterial Factor(s) to Cellulose Phosphate

Preliminary experiments on the adsorption of antibacterial material from HSP by cellulose phosphate and the elution of this material with sodium citrate were conducted. A ratio of 2.5 cm^3 HSP (warmed to 37^o to aid pipetting) to 3.0 cm^3 CP slurry was used and the procedure continued as outlined in Methods 2.3.3.2.

2.3.3 Standard Procedure (Fig.5)

2.3.3.1 Large Batch Treatment

Routinely, 50 or 100 cm^3 of BSP or pancreas extract were treated with cellulose phosphate. The treatment was carried out at room temperature. All centrifugations were at 12,000 x g for 15 min at 0^o. The treatment was as follows:

1. Bovine seminal plasma (100 cm^3) or pancreas extract (100 cm^3) was added to 120 or 20 cm^3 of cellulose phosphate in 0.1M sodium phosphate, pH 7.4, respectively, and held for 10 min with gentle stirring.

2. The mixture was divided into 8 aliquots and centrifuged in 30 x 100 mm tubes to obtain the first supernatant.
3. Each CP pellet was washed with 10 cm^3 of 0.1M phosphate buffer, pH 7.4 (80 cm^3 total) and centrifuged to obtain the first buffer wash.
4. Each pellet was then washed twice with 30 cm^3 of 0.2M NaCl. The suspensions were mixed occasionally for 15 min and then centrifuged to obtain two NaCl washes.
5. The pellets were washed again with 10 cm^3 of 0.1M phosphate buffer, pH 7.4 (80 cm^3 total) and centrifuged to obtain the second buffer wash.
6. The antibacterial factor was then eluted from the cellulose phosphate by washing each pellet with 7.5 cm^3 of 1.6M sodium citrate. The suspensions were mixed occasionally for 15 min, pooled in a beaker and diluted with 240 cm^3 of 0.01M sodium phosphate buffer, pH 7.4. Then the mixture was centrifuged to obtain a final supernatant (eluate) of about 300 cm^3.

2.3.3.2 Small Batch Treatment

The volumes used in the large batch preparation were based on a small batch procedure involving 2.5 cm^3 of BSP or pancreas extract per 3.0 or 0.5 cm^3 of CP slurry respectively; a 2.0 cm^3 buffer wash; two, 7.5 cm^3 NaCl washes (adjusted to 6.0 cm^3 for ease of handling larger batches); a second 2.0 cm^3 buffer wash and a final 1.5 cm^3 sodium citrate wash diluted 5 times with distilled water or buffer.

2.4 Ethanol Precipitation of the Antibacterial Factors (Fig.5)

2.4.1 Bovine Seminal Plasma Eluate from Cellulose Phosphate

Initially, the ethanol precipitation profile of a final eluate

prepared from cellulose phosphate treated with BSP was determined at 25^{o} and 0^{o}. All further precipitations were carried out at 0^{o} in an ice bath.

A calculated volume of chilled absolute ethanol was added dropwise to a known volume of final eluate in 0.01M sodium phosphate buffer, pH 7.4,with constant stirring. Following the addition of ethanol the mixture was allowed to equilibrate for 15 min at 0^{o}. The suspension was centrifuged at 12000 x g for 15 min at 0^{o} to obtain the precipitate; this was lyophilized in a pre-weighed container.

The terminology used throughout the study defines a 1:1.5 precipitate, for example, as one obtained from a solution which is 1 part aqueous and 1.5 parts ethanol. Following lyophilization the precipitate was weighed and resuspended to a known volume with distilled water, such that the citrate concentration was less than 0.5M. For example, the 1:2 ppt. was usually resuspended to 75 to 85% of the volume of eluate used in the precipitation; the 1:4 ppt. to a volume 5 to 25% that of the initial eluate volume. The lyophilization step was sometimes omitted and the precipitate was resuspended directly in distilled water.

The supernatant was then treated in the same manner and the process was continued until the desired ethanol concentration was obtained. Routinely, 20.0 cm^3 of the final aqueous-ethanol supernatant was lyophilized and resuspended in 1.0 cm^3 of distilled water. The antibacterial activities and the protein concentrations of the various fractions were determined.

A sample calculation for the addition of ethanol follows:

1. initial eluate volume = 20 cm^3
2. for a 1:1.5 ppt. add 1.5 x 20 = 30 cm^3 ethanol
3. supernatant collected after centrifugation may be 45 cm^3, of

which 1 part in 2.5 is aqueous = 45/2.5 = 18 cm^3

4. for a 1:3.5 ppt. require 3.5 x 18 = 63 cm^3 ethanol, but 45 - 18 = 27 cm^3 ethanol is present in the recovered supernatant. Therefore, add 63 - 27 = 36 cm^3 ethanol.

In several cases precipitates were dissolved and reprecipitated with ethanol to obtain preparations with higher specific activites and lower citrate concentrations. Precipitates obtained in this manner were designated ppt^1.

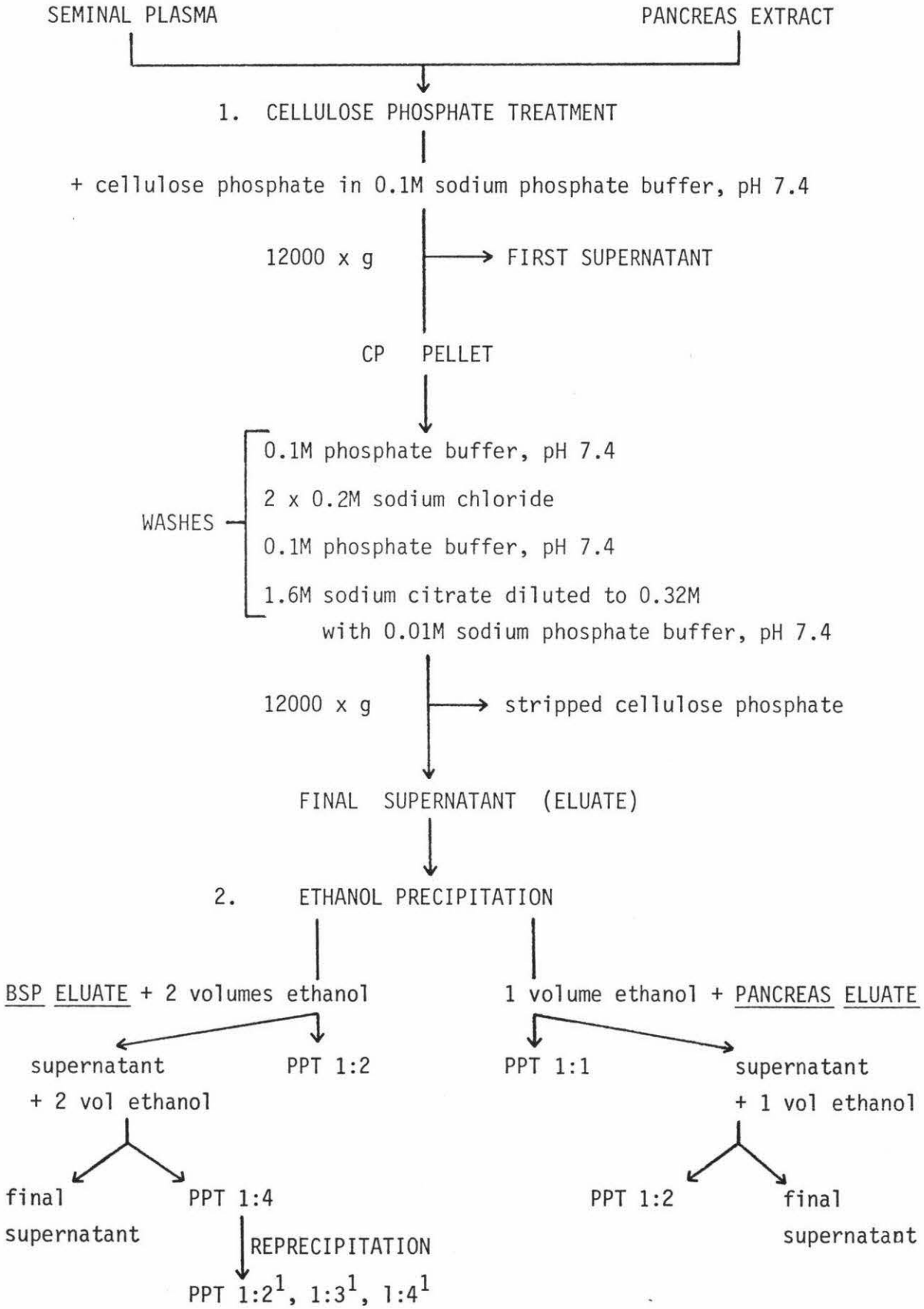
The standard ethanol precipitation of an eluate from BSP treated cellulose phosphate involved a 1:2 and a 1:4 precipitation. In several cases,a reprecipitation of an aqueous solution of a 1:4 precipitate involved a $1:2^1$, $1:3^1$ and $1:4^1$ precipitation.

2.4.2 Pancreas Eluate from Cellulose Phosphate

The effect of pH on the ethanol precipitation of a 1:2.5 precipitate, resuspended in 90 cm^3 of distilled water and divided into 3 aliquots, was studied. To each aliquot, 30 cm^3 of 0.02M sodium phosphate buffer, pH 5.8, 7.0 or 8.0, was added to give a final buffer concentration of 0.01M. The ethanol precipitation over the range 1:1 to 1:4 was done as in Methods 2.4.1.

The standard ethanol precipitation of an eluate from pancreas treated cellulose phosphate, involved a 1:1 and 1:2 precipitation, unless otherwise indicated. In several cases,the aqueous 1:2 precipitate was dialyzed against 0.01M phosphate buffer, pH 7.4, or distilled water for 4 to 6 h to reduce the citrate concentration.

Fig. 5 Standard preparation of antibacterial fraction



2.5 Ammonium Sulphate Fractionation of BSP and Pancreas - Cellulose Phosphate Eluates

Fifty cm^3 of final eluate, prepared from cellulose phosphate treated with bovine seminal plasma or pancreas extract and diluted with 0.01M sodium phosphate buffer, pH 7.4, was transferred to a 250 cm^3 beaker. The calculated quantity (Dawson et al., 1972) of crystalline $(NH_4)_2SO_4$ was then added at 0^o, with constant stirring, to give the required saturation. Each solution was stirred for 15 min. Then the precipitate was equilibrated with its supernatant for 15 min before centrifugation at 12000 x g for 10 min at 0^o. The supernatant was collected and was again treated with ammonium sulphate, and the process was continued over the 20 to 70% saturation range. Each precipitate was washed into a preweighed bottle with several cm^3 of distilled water, lyophilized and resuspended to a known volume.

A portion of each precipitate solution was dialyzed against 100 volumes of 0.01M sodium phosphate buffer, pH 7.4, for 16 h. The buffer was replaced and the samples were dialysed for 4 more hours to reduce the ammonium sulphate concentration to less than 0.15%, which is tolerable in the Lowry protein determination (Lowry et al., 1951). Protein determinations were carried out following dialysis. Antibacterial activity was determined before and after dialysis of each sample.

3. Characterization of Antibacterial Factors from Bovine Seminal Plasma and Pancreas

3.1 Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis

With minor modifications, the preparation of samples and the electrophoresis procedure followed that of Weber et al. (1972).

3.1.1 Preparation of Protein Samples

Protein solutions were treated to bring about complete denaturation, and the reduction of disulphide bonds; one volume of a protein solution was added to nine volumes of denaturing buffer (0.1M Tris - glycine buffer, pH 8.9, containing 1.0% SDS, 1.0% β - mercaptoethanol and 6.0M urea) (Materials 4.3) in a small vial, which was capped and heated for 2 min in a 100^{o} bath. The final protein concentration ranged from 0.05 to 1.0 mg/cm^3.

3.1.2 Preparation of Gels

For 10% gels the following solution was freshly prepared: 2.5 g of acrylamide (J.T. Baker Chem. Co.), 0.084 g of N, N^1-methylene bisacrylamide (BDH) and 25 µl of N, N, N^1, N^1-tetramethylethylenediamine (TEMED) (Sigma) diluted to 25.0 cm^3 with gel buffer (0.1M Tris - glycine, pH 8.9, 0.1% SDS) (Materials 4.3), and polymerized with 20 mg ammonium persulphate (AnalaR). The solution was mixed and glass gel tubes, 75 mm long and 6 mm inside diameter, were filled with about 2 cm^3 of the gel solution, which was carefully overlaid with buffer using a syringe. All gel tubes were acid washed, rinsed in deionized distilled water and oven dried prior to use. The gels solidified after 10 to 20 min.

3.1.3 Electrophoresis

The two compartments of the electrophoresis apparatus were filled with gel buffer; the gels were loaded and pre-electrophoresis was conducted for 15 min at 2mA per gel to remove impurities from the gels. The upper reservoir was then replaced with fresh buffer.

For each gel, 10 µl of tracking gel (0.05% bromphenol blue in water), 1 drop of glycerol and an appropriate amount of denatured protein sample were mixed on a small square of parafilm. The total sample volume was from 25 to 150 µl and contained 1 to 125 g of protein, but in most cases 2 to 20 µg of protein were applied to the gels with a micropipette.

Electrophoresis was performed (with the anode in the lower chamber) at room temperature since SDS is insoluble in reservoir buffer below 15^{o}. A constant current of 2mA per gel was applied for 15 min and then 4mA per gel until the marker dye approached the bottom of the gel, about 60 to 70 min.

3.1.4 Staining and Destaining

After electrophoresis the gels were removed from the tubes and the dye band was marked at the centre with a fine wire. Gels were stained at room temperature for 6 to 16 h in 0.025% Coomassie Brilliant Blue in destain-solution. They were destained by diffusion in a solution of 7 cm^3 glacial acetic acid, 25 cm^3 methanol and water to 100 cm^3. Frequent changes of the destain-solution facilitated the removal of excess dye from the gels. Gels were stored in 7.5% glacial acetic acid solution and scanned in a Beckman Acta III spectrophotometer at 600 nm with a 0.05 mm slit fitted into the gel scanner. A chart expansion of 5 nm/in, a scan speed of 0.1 nm/sec and a slit position of 1.5 cm/min were used.

3.1.5 Determination of Molecular Weight

Mobilities, calculated as: $\frac{\text{distance of protein migration}}{\text{distance of dye migration}}$, were plotted on a semi-logarithmic scale against the known molecular weights of standard marker proteins (bovine serum albumin, ovalbumin, β- lactoglobulin, α - lactalbumin, lysozyme and cytochrome c) (Weber et al., 1972) electrophoresed simultaneously in adjacent gels.

3.2 Native Polyacrylamide Gel Electrophoresis

Polyacrylamide gels (7%) were prepared as described by Kersters and De Ley (1975) in 75 mm tubes with an inner diameter of 6 mm. Gels were polymerized in a constant 24^{o} bath for at least 2 h. The gel buffer, 0.064M Tris-HCl, pH 8.7, was circulated throughout the apparatus at 8 to 9^{o} during electrophoresis. A 10 µl sample containing 50 to 100 µg of protein in 5% (w/v) sucrose was applied to each gel. Electrophoresis was performed with the anode in the lower chamber at a constant current of 1.25mA/gel for 15 min, followed by 3.8mA/gel until a bromphenol blue marker had moved 43 mm through the gel, about 110 min. The gels were removed from the tubes, and stained and destained by the procedure described in Methods 3.1.4. This technique was used for a preliminary comparison of the components of bovine seminal plasma, NaCl washes, final eluates after cellulose phosphate treatment and ethanol precipitated material.

3.3 Ultraviolet Absorption

The ultraviolet absorption scans (200 to 450 nm) of three different BSP preparations (0.9, 0.9 and 4.5 mg protein/cm^3) and one pancreas preparation (1.2 mg protein/cm^3) were determined on a Unicam SP800 spectrophotometer.

3.4 Amino Acid Analysis

The amino acid analyses were performed on a Beckman 120 C Analyzer after hydrolysis of a BSP preparation (ppt 1:3[1], 0.5 mg protein/cm^3, 18400 units/mg) for 24, 48 and 72 h at 110^o with 6N HCl.

3.5 Determination of Lysozyme Activity

The enzymic activity of lysozyme was determined by the colorimetric assay of Otaki and Kimura (1975) based on the hydrolysis of the synthetic substrate, 3,4-dinitrophenyl-tetra-N-acetyl-β -D-chitotetraoside (Koch Light Lab. Ltd.).

The 3,4-DNP glycoside solutions employed had concentrations of 0.5 mg and 1.0 mg per cm^3 of 0.1M citrate buffer, pH 6.0. The lysozyme solutions had final concentrations of 0.05 and 0.1 mg per cm^3 of citrate buffer. The purified BSP and pancreas preparations were diluted to 0.1 mg/cm^3 with citrate buffer.

One cm^3 of enzyme solution was preincubated for 3 min at 40^o in a 0.5 cm cuvette contained in the constant temperature compartment of a Unicam SP 1800 spectrophotometer. The substrate solution was also preincubated at 40^o. The assay was performed by adding 0.1 cm^3 of substrate solution to 1.0 cm^3 of enzyme solution. The cuvette was capped, mixed by inversion and the increase in absorbance was measured spectrophotometrically at 400 nm over 30 min against a blank containing 1.0 cm^3 of citrate buffer and 0.1 cm^3 of substrate solution.

3.6 Determination of Haemolytic Activity

3.6.1 Plate Assay

The haemolytic activities of bovine seminal plasma and a purified BSP preparation were determined on 5% blood agar (Materials 3.2).

Haemolysis of bovine, chicken, horse, sheep and human blood was compared. Wells, 4 mm in diameter, were cut; the agar was removed and the wells filled with two drops of sample (less than 0.05 cm^3). The plates were incubated at 37^o for 20 h and clear zones of haemolysis were measured with calipers.

3.6.2 Tube Assay

Haemolytic activity was determined by the method of Mitsui et al. (1973). A 1% human red blood cell suspension was prepared by washing the cells twice with 0.14M NaCl, and twice with 0.2M borate buffer, pH 7.6 (Taguchi and Ikezawa, 1975) containing 0.008M NaCl and 0.01M $CaCl_2$. The cells were resuspended in borate buffer and diluted to obtain a 1% suspension. A 1% suspension was defined as that which gave an absorbance at 550 nm of 0.47 ± 0.01 when 1.5 cm^3 of the suspension was added to 4.5 cm^3 of water. The latter represented a completely haemolyzed solution.

To determine haemolytic activity 3.0 cm^3 of a freshly prepared cell suspension was added to 3.0 cm^3 of a sample diluted with borate buffer and the mixture was incubated at 37^o for 30 min. The tubes were immediately immersed in an ice-water bath for 30 min and the supernatant was collected by centrifugation at 3000 rpm for 15 min. The absorbance of the supernatant was determined at 550 nm and the haemolytic activity was calculated. The haemolytic activity of the test solution was defined as one unit when the absorbance of the supernatant was the same as that of a completely haemolyzed solution of 1.5 cm^3 of a 1% red cell suspension as described above.

3.7 Determination of Phospholipase Activity

Egg yolk plates (Materials 3.2) as described by Habermann and Hardt (1972) were used to detect phospholipase activity in whole seminal plasma, crude pancreas extracts, final eluates after cellulose phosphate treatment and ethanol precipitated BSP material. Wells, 4 mm in diameter, were cut and the agar removed by suction. The wells were filled with two drops of sample, and the plates were incubated at 37^{o} or 50^{o} for 20 h. The effects of less heat stable enzymes, such as trypsin and chymotrypsin, are eliminated at the higher temperature (Habermann and Hardt, 1972).

The sample diffused into the gel and either cleared the egg yolk (phospholipase A) or increased its turbidity (phospholipase C). A mixture of phospholipase A and B would be indicated by a clear zone with a turbid centre if phospholipase A was in a greater proportion than phospholipase B (Habermann and Hardt, 1972). All zones were measured to the nearest 0.1 mm with Vernier calipers.

3.8 Trypsin Sensitivity

The effect of trypsin on the antibacterial activity of purified BSP material (11,380 units/mg) and pancreas material (11,190 units/mg) was determined. Initially, 0, 10, 100 or 1000 μg of trypsin in 0.1 cm^3 of water was added to 1.2 mg of purified BSP material in 0.4 cm^3 of water in a screw cap bottle. Each solution was mixed and 0.25 cm^3 was quickly transferred to a second screw cap bottle and promptly heated in a boiling water bath for 10 min to destroy trypsin activity (Hirsch and Dubos, 1954). The remainder of each solution was incubated at 37^{o} for 30 min. The antibacterial activity of the solutions was determined in duplicate and compared to that of a control without trypsin.

Trypsin at concentrations as high as 10 mg/cm^3 exhibited no antibacterial activity in the agar diffusion assay.

In succeeding runs, 0, 1 or 10 μg of trypsin in 0.1 cm^3 of water was added to either 0.4 or 0.5 cm^3 of purified antibacterial material (0.5 mg or 1.2 mg of BSP material and 0.5 mg of pancreas material was used per reaction mixture) and the procedure continued as above.

3.9 Heat Stability

The thermostability of a given preparation was tested in distilled water by heating 0.3 cm^3 samples in a 5 cm^3 screw cap bottle for 10 min by immersion in a water bath ranging from 50 to 100° in 10° stages. Following heating, the bottles were plunged into an ice-water bath for 5 min. The antibacterial activity was determined for each sample in duplicate and compared with that of an untreated sample. The effect of autoclaving on the antibacterial activity of a purified preparation was determined by autoclaving a 0.6 cm^3 sample in a 5 cm^3 screw cap bottle for 15 min at 120°.

3.10 Inhibition of Antibacterial Activity by Deoxyribonucleic Acid

The effect of calf thymus DNA, an anionic polymer, on the antibacterial activity of purified BSP material (11,380 units/mg) and pancreas material (11,190 units/mg) was determined. A reaction mixture containing 0.25 or 0.5 cm^3 of purified material (0.25 mg or 0.5 mg protein) and 0.5 cm^3 of DNA solution, pH 7.0, (0.5 mg) was prepared and the volume made to 1.0 cm^3 with distilled water. Controls, without DNA, and with DNA alone, were included. All solutions were incubated at 37° for 15 min and then chloroform treated as described in Methods 1.1.2.

The antibacterial activity of each solution was determined using the

MIC broth dilution method (Methods 1.1.2). Six serial two-fold dilutions were prepared from each reaction mixture with the first tube in the series containing either 50 or 100 µg of purified antibacterial protein. The MIC of untreated material was compared with that of DNA treated material. A deoxyribonucleic acid control exhibited no antibacterial activity.

3.11 Ethanol Precipitation Following Sample Acidification

3.11.1 BSP Precipitate 1:2

The reprecipitation of an aqueous solution of a 1:2 precipitate from BSP was studied under different conditions. Firstly, the aqueous solution was precipitated directly with ethanol. The profile obtained was compared to that of a solution which had been adjusted to pH 2.0 by the dropwise addition of concentrated HCl, allowed to equilibrate for 15 min, and then readjusted to pH 7.4 with 6M NaOH before precipitating with ethanol.

Ethanol reprecipitation following acidification was repeated on three different 1:2 precipitate solutions yielding $1{:}2^{1}_{acid}$, $1{:}4^{1}_{acid}$, and in the first case $1{:}7^{1}_{acid}$ precipitates. In the first run an aliquot of the supernatant, following each addition of ethanol, was lyophilized and concentrated 4 to 8 times, and the protein and activity were determined. In the second and third runs the entire supernatant, following each precipitation, was reprecipitated.

In the third run an acetone precipitation was performed at 0^{o} on a 20 cm^3 aliquot of the final supernatant obtained from the $1{:}4^{1}_{acid}$ precipitation. Acetone at 0^{o} was added dropwise and a $1{:}3^{1}$ (75% acetone) precipitate was obtained, lyophilized and resuspended to a known volume with distilled water. The addition of a further 0.5 cm^3 of acetone to

the final supernatant resulted in no further precipitation. An aliquot of the final supernatant was lyophilized, concentrated 20 times and tested for antibacterial activity.

3.11.2 BSP Precipitate 1:4

The citrate concentration of an 8.0 cm^3 aqueous solution of a 1:4 precipitate was adjusted from 3.3 mg/cm^3 to 65.0 mg/cm^3 by the addition of 0.49 g of sodium citrate to yield a solution with a similar citrate concentration to that of an aqueous solution of a 1:2 precipitate. The solution was adjusted to pH 2.0 with concentrated HCl, allowed to equilibrate for 15 min, and then readjusted to pH 7.4 with 6M NaOH. The solution was precipitated with ethanol to yield $1:2^1_{acid}$ and $1:4^1_{acid}$ precipitates.

3.12 Ion Exchange Chromatography on Cellulose Phosphate

A cellulose phosphate slurry equilibrated with 0.05M Tris-HCl buffer, pH 7.4, was evacuated for 30 min. A cellulose phosphate column was then packed to a height of 17.5 cm in a Wright polycarbonate column (no dead space), 30 cm high with an inner diameter of 1.6 cm. Each sample was applied to a column having a bed volume of approximately 35 cm^3 and equivalent to 42 g wet weight of cellulose phosphate as determined routinely during cellulose phosphate preparation (Methods 2.3.1). Five cm^3 fractions were collected at a flow rate of 5 cm^3/10 min.

The sample was run into the column which was then washed with 70 cm^3 of 0.05M Tris-HCl buffer, pH 7.4. The column was developed with a linear gradient of sodium citrate in Tris buffer (0 to 0.5M or 0.1 to 0.4M, 70 cm^3 of each solution). At the conclusion of the gradient the column was washed with 70 cm^3 of 1.0M sodium citrate in Tris buffer.

The absorbance at 280 nm and the conductivity were recorded for each fraction. The conductivity of samples was read on ice using a Copenhagen radiometer conductivity meter, and converted to molarity using sodium citrate in 0.05M Tris-HCl buffer, pH 7.4,as a standard (Fig. 6). Fractions containing protein, as indicated by the absorbance at 280 nm, were assayed for antibacterial activity.

Solutions of BSP 1:2 and 1:2[1] precipitates were fractionated chromatographically on cellulose phosphate. A large volume of the BSP 1:2 ppt solution was first dialyzed against 20 volumes of distilled water for 4 h to reduce its citrate concentration. The sample was then concentrated and analyzed for activity, protein and citrate. Dialysis reduced the citrate concentration from 54 mg/cm^3 (0.3M) to 20 mg/cm^3 (0.1M).

Following the initial column chromatography of dialyzed, BSP ppt 1:2,several small volume batch adsorptions (0.5, 1.0 or 2.0 cm^3 sample + 3.0 cm^3 CP slurry) were performed. The adsorption of activity from BSP and dialyzed, BSP ppt 1:2 to cellulose phosphate equilibrated in 0.1M sodium phosphate, pH 7.4,or 0.05M Tris-HCl buffer, pH 7.4,was compared. The first supernatants were collected by centrifugation and assayed. Each CP pellet was washed with 2.0 cm^3 of the appropriate buffer. A final wash of 1.6M sodium citrate was used to elute the adsorbed activity from the CP pellets. The final eluates were collected and assayed.

3.13 Serology

3.13.1 Antigen Preparation

Alcohol precipitated BSP material with high specific activity was dissolved in water to a known protein concentration. Purified pancreas

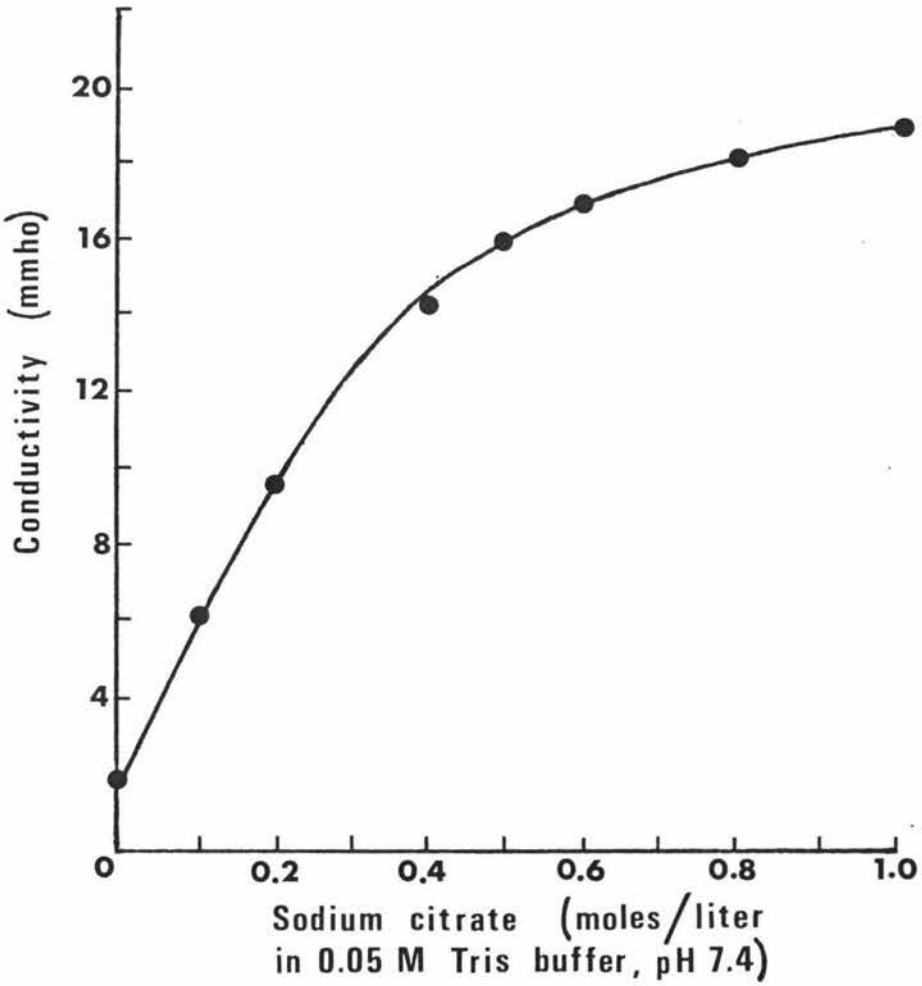


Fig. 6 Conductivity of sodium citrate in 0.05M Tris-HCl buffer, pH 7.4

preparations were dialyzed against 30 volumes of 0.01M sodium phosphate buffer, pH 7.4 for 5 h, concentrated initially against polyethylene glycol 6000 (BDH) and then lyophilized and resuspended to a known volume in water. The second pancreas preparation was further dialyzed against 200 volumes of deionized distilled water for 2.5 h in an attempt to remove the cause of a precipitation reaction, which occurred during the Lowry protein determination on the sample. This was not successful however. Vallejo and Lagunas (1970) noted the formation of a white precipitate due to potassium ions in concentrations greater than or equal to 12 mM. The precipitate was removed by centrifugation and the absorbance of the supernatant was recorded; no difference in color was observed. The same was done for the two pancreas preparations, although the cause of the precipitate in the presence of the Lowry reagents was unknown.

Table I. Antigens used for the immunization of rabbits

RABBIT	ANTIGEN	SPECIFIC ACTIVITY (units/mg protein)
A	BSP ppt 1:4[1]	17,370
B	BSP ppt 1:3[1]	16,860
C,D	pancreas ppt 1:2 (1)	15,910
C,D	pancreas ppt 1:2 (2)	11,190

3.13.2 Immunization Schedule

1. The rabbits were bled prior to the start of immunization.
2. On day one 0.5 mg protein (Table I) contained in a 50:50 antigen-complete Freund's adjuvant (Difco) mixture was injected intramuscularly into each hind leg.
3. Four weeks later 0.5 mg protein was injected intravenously into an ear.
4. One week later 0.5 mg protein was again injected intravenously into the same ear.
5. The rabbits were then bled one week later (six weeks after the start of the schedule).
6. One week later rabbits C and D were each injected intravenously with 0.5 mg of a second pancreas preparation (2) in an attempt to increase their antibody titre. Rabbits A and B were bled again.
7. Ten days later all four rabbits were bled.

3.13.3 Antisera Preparation

Blood was collected in 150 x 19 mm tubes by exsanguination from an ear vein. The blood was allowed to clot at 37^{o} for 6 h and then held at 4^{o} overnight for clot contraction. The serum was pipetted off, centrifuged at 4000 rpm for 10 min and the supernatant was stored at -16^{o}.

3.13.4 Immunodiffusion Studies

Immunodiffusion studies were based on the method of Ouchterlony (1967). Antigen and antibody samples were added to wells with diameters of 4 or 5 mm, which were cut in immunodiffusion agar (Materials 3.2) with a metal bore. All sample dilutions were made in

0.14M NaCl. Immunodiffusion plates were incubated at room temperature and observed over a period of 3 days for the development of precipitin lines. A 24 h incubation yielded the clearest precipitin patterns in most cases. All immunodiffusion studies in which no precipitin reaction was observed were repeated with variations in well patterns and well sizes.

3.13.4.1 Serological Identification of Protein Fractions

Serological studies were directed towards the identification of specific protein fractions recovered by column chromatography and gradient elution of cellulose phosphate; these fractions were identified with specific precipitin lines developed in double-diffusion agar plates. For these studies the control antigens were run against a mixture of one volume of antiserum B plus one volume of "column-antigen", and a mixture of one volume of antiserum B plus one volume of 0.14M NaCl.

3.13.5 Neutralization of Antibacterial Activity by Antisera

The neutralization of the antibacterial activity of purified BSP material (10,560 units/mg and 8740 units/mg) by antisera A and B was attempted using the MIC broth dilution technique (Methods 1.1.2). Four sets of serial two-fold dilutions of each antibacterial substance were prepared in nutrient broth. To each of the first three sets, 0.05 cm^3 of normal rabbit serum, 0.05 cm^3 of antiserum A and 0.1 cm^3 of antiserum B were added per tube, respectively. The final set served as a control.

The tubes were mixed, inoculated with 0.05 cm^3 of an overnight culture of M. lysodeikticus and incubated at 37^o for 24 h. As the addition of antiserum to nutrient broth resulted in turbidity, this

made the evaluation of growth difficult; growth was monitored by streaking onto nutrient agar plates, which were incubated at 37^{o} for 24 h.

The results indicated that in the presence of normal serum and antiserum A or B, the antibacterial activity of the purified material was not neutralized, but was, instead, greater than that of the purified material alone. The antibacterial activity of the antiserum was therefore determined at various dilutions in 0.14M NaCl using the agar diffusion technique (Methods 1.1.1). At a dilution of 1:20 neither antiserum exhibited detectable antibacterial activity.

The neutralization of the antibacterial activity of histones f1 and f2a, and purified BSP material (10,560 units/mg) was then attempted by the addition of one volume of antiserum A or B, diluted 1:10 with saline, to one volume of antibacterial material. The final mixtures contained antiserum diluted 1:20 and final protein concentrations of histones f1 and f2a, and BSP material of 0.85, 0.5 and 0.45 mg/cm^3 respectively. The antibacterial activity of these solutions was determined by agar diffusion and was compared with that of one volume of antibacterial material diluted with one volume of 0.14M NaCl.

RESULTS

RESULTS

1. Antibacterial Activity of Bovine Seminal Plasma

1.1 Agar Diffusion (Methods 1.1.1)

Table II summarizes the antibacterial activities and protein concentrations of different pooled batches of bovine seminal plasma. The average antibacterial activity of twelve individual batches of BSP was 28,700 units/cm^3, and the average protein concentration of six batches was 90 mg/cm^3. A considerable range in protein concentration from 47 to 160 mg/cm^3 was observed. Throughout this study, specific activity, defined as antibacterial units* /mg protein, was used as an index of the purity of a given preparation. The average specific activity of BSP was 320 units/mg protein.

Fig. 7 records the antibacterial activity of bovine seminal plasma at various dilutions in distilled water. Unlike crude BSP, a plot of the antibacterial activity of purified BSP material (8970 units/mg) at various dilutions in distilled water gave a linear response and was proportional to dilution (Fig. 8).

The failure to observe a linear response of activity with dilution of whole BSP should be compared and related to a later observation, that the total activity which can be accounted for during the purification procedure is greater than the initial activity observed.

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* unit defined in Methods 1.1.1.

Table II. Antibacterial activity and protein concentrations of whole bovine seminal plasma and pancreas extracts

	Activity ($units/cm^3$)	Activity is the average of x determinations where x equals:	Protein (mg/cm^3)
BSP Batch			
1	26,500	3	
2	33,500	3	
3	35,000	3	
4	23,500	6	
5	20,173	39	
6	28,625	12	73.6
7	30,400	3	118.0
8	42,000	3	159.5
9	33,000	3	82.8
10	21,250	3	58.6
11	19,250	3	
12	31,000	6	46.8
Pancreas Extract			
1	22,250	3	16.4
2	24,500	3	10.5

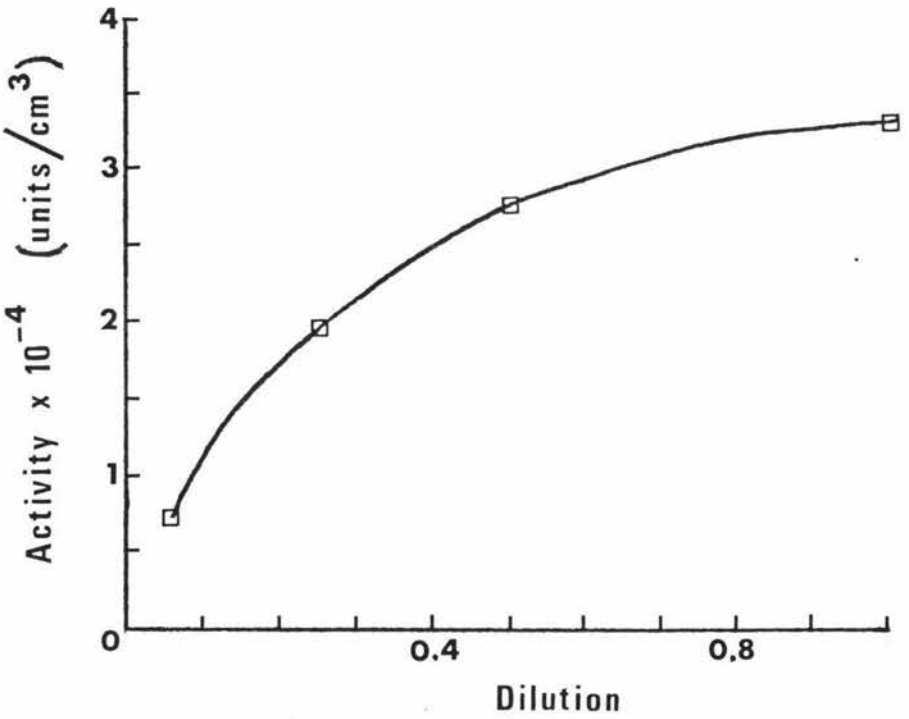

Fig. 7 Antibacterial activity of whole bovine seminal plasma at various dilutions

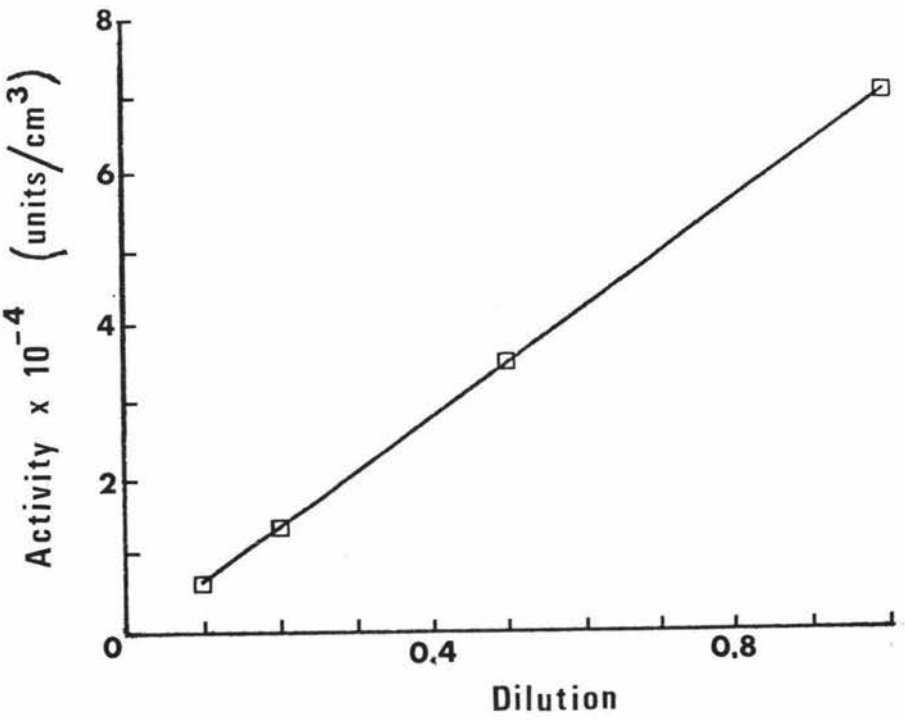

Fig. 8 Antibacterial activity of purified BSP material at various dilutions

2. Antibacterial Activity of the Histones

The antibacterial activities of four histone fractions (f1, f2a, f2b and f3) as described by Johns (1964) were determined and the results are presented in Table III. Histone solutions were prepared gravimetrically at 10.0 mg/cm^3 distilled water, and activity and protein were determined in solutions diluted ten-fold with water.

Table III. Antibacterial activity of the histones

Histone	Description	Activity (units/cm^3)	Protein (mg/cm^3)	Specific Activity (units/mg)
f1	lysine rich	1760	1.1	1600
f2a	slightly lysine rich	1175	1.0	1180
f2b	slightly lysine rich	480	1.2	400
f3	arginine rich	not detected	0.9	-

The specific activity of the most active histone fraction was less than one-tenth of that which has been observed for the most active BSP preparations. Evidence will also be provided that the ultimate specific activity of the BSP factor may approach 34,000 or 21 times that observed for the f1 histone preparation.

3. Preparation of Crude Pancreas Extract

3.1 Determination of Optimal Conditions for Recovery of Activity from Bovine Pancreas

Initially, the extraction of antibacterial material from pancreas was attempted using 0.1M sodium phosphate buffer, pH 7.0; 1.0M sodium citrate in phosphate buffer, pH 7.0; and 1.0M sodium citrate, pH 2.0.

The results, presented in Table 1VA, demonstrate that the best recovery of antibacterial activity is obtained with citrate under acidic conditions. Seventeen and thirteen times more activity was recovered with acid-citrate, than with buffer or buffered-citrate respectively.

A further study of the acid-citrate extraction was undertaken to determine the importance of the acid in the extraction procedure. The results (Table IVB) indicate that acid alone, like citrate alone, was not sufficient to obtain the maximum yield of antibacterial activity. Also, a comparison between 0.5M and 1.0M citrate showed 0.5M citrate, pH 2.0, to be more effective than 1.0M citrate, pH 2.0, for the isolation of active material from pancreas.

3.2 Dialysis of Crude Pancreas Extracts

The citrate extracts were dialyzed, in an effort to reduce the citrate concentration in the extracts to acceptable levels for treatment with cellulose phosphate for the further purification of the antibacterial substance. Several variations of the dialysis conditions were studied.

Extracts, which were obtained with 1.0M citrate in phosphate buffer, pH 7.0, and 1.0M citrate, pH 2.0, then neutralized, were dialyzed against 400 volumes of distilled water at 4^{o} for 23 h with stirring. The water was replaced and the samples were dialyzed for a further 20 h. The per cent activity lost was calculated as follows:

$$100 - \left(100 \times \frac{\text{activity after dialysis} \times \text{volume after dialysis}}{\text{activity before dialysis} \times \text{volume before dialysis}}\right)$$

Following dialysis, more than 86% of the buffer-citrate extract activity and 66% of the acid-citrate extract activity were lost. Similarly, the dialysis of a neutralized, 0.5M acid-citrate extract, and a second 1.0M acid-citrate extract under the same conditions, resulted in the loss of 71% of the activity.

Table IV. Preparation of pancreas extract

	Wt. Tissue (g)	Extract Volume After Neutralization (cm^3)	Activity (units/cm^3)	Antibacterial Units/g Tissue
IVA. Initial Study				
1. 0.1M phosphate buffer, pH 7.0	17.1	22.0	740	950
2. 1.0M citrate in phosphate buffer, pH 7.0	22.4	12.0	2,400	1,290
3. 1.0M citrate, pH 2.0	28.0	33.0	13,750	16,450
IVB. Effect of Acid				
1. distilled water, pH 2.0	42.0	45.0	640	690
2. 0.5M citrate, pH 2.0	42.0	45.0	24,500	26,250
3. 1.0M citrate, pH 2.0	42.0	43.0	18,500	18,940
IVC. Variation of Citrate Concentration				
1. 0.1M citrate, pH 2.0	32.0	27.5	16,500	14,180
2. 0.2M "	31.0	22.0	23,500	16,680
3. 0.3M "	24.0	18.8	18,000	14,100
4. 0.4M "	24.0	19.5	17,000	13,810
5. 0.5M "	25.0	20.0	19,250	15,400

Finally, the dialysis of 0.5M and 1.0M citrate extracts against 400 volumes of 0.05M sodium phosphate buffer, pH 7.0, for 45 h, with one change of buffer at 24 h, resulted in the loss of 13% and 42% activity respectively. Thus, dialysis against phosphate buffer resulted in lower losses of activity than did dialysis against water.

However, due to the loss of activity following dialysis, the use of citrate concentrations, less than 0.5M, for the isolation of active material from pancreas, was studied. A comparison of extractions using 0.1 to 0.5M sodium citrate, pH 2.0, is presented in Table IVC. The yield of antibacterial activity per gram of pancreatic tissue did not differ significantly in the range 0.1 to 0.5M citrate. However, the 0.2M citrate extraction resulted in a slightly greater yield, and therefore, 0.2M citrate, pH 2.0, was chosen for all standard preparations of crude pancreas extract.

3.3 Antibacterial Activity of Pancreas Extract (Table II)

The average activity of two crude pancreas extracts, which were prepared as described in Methods 2.3.3.1, was 23,400 units/cm^3, and the average protein concentration was 13 mg/cm^3. The average specific activity of the crude pancreas extract was 1800 units/mg protein.

Fig. 9 illustrates the antibacterial activity of two neutralized, 0.5M citrate, pH 2.0, extracts at various dilutions in 0.05M phosphate buffer, pH 7.0. One extract was dialyzed against 0.05M phosphate buffer, pH 7.0, for 45 h.

Fig. 10 shows the antibacterial activity of a neutralized, 0.2M citrate, pH 2.0, extract at various dilutions in 0.05M phosphate buffer, pH 7.0.

Unlike the dilution of BSP, the dilution of pancreas extract results in a linear relationship between activity and dilution.

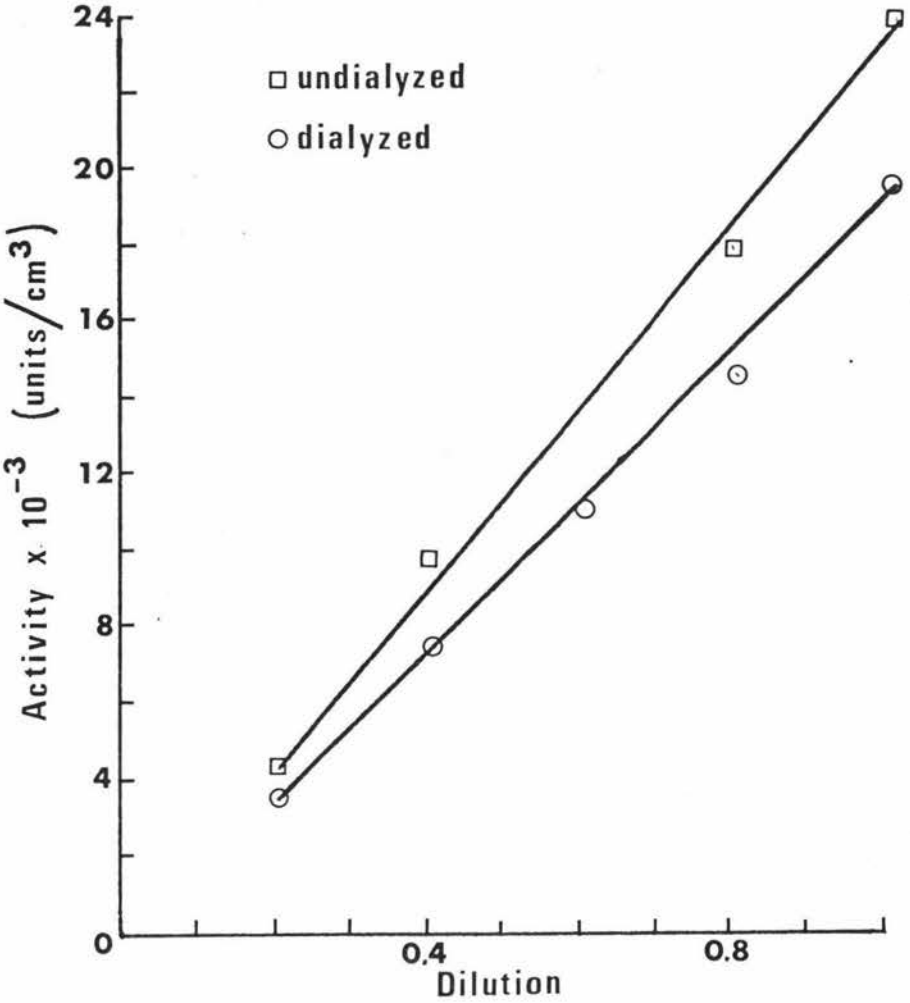

Fig. 9 Antibacterial activity of neutralized, 0.5M citrate, pH 2.0, pancreas extracts at various dilutions

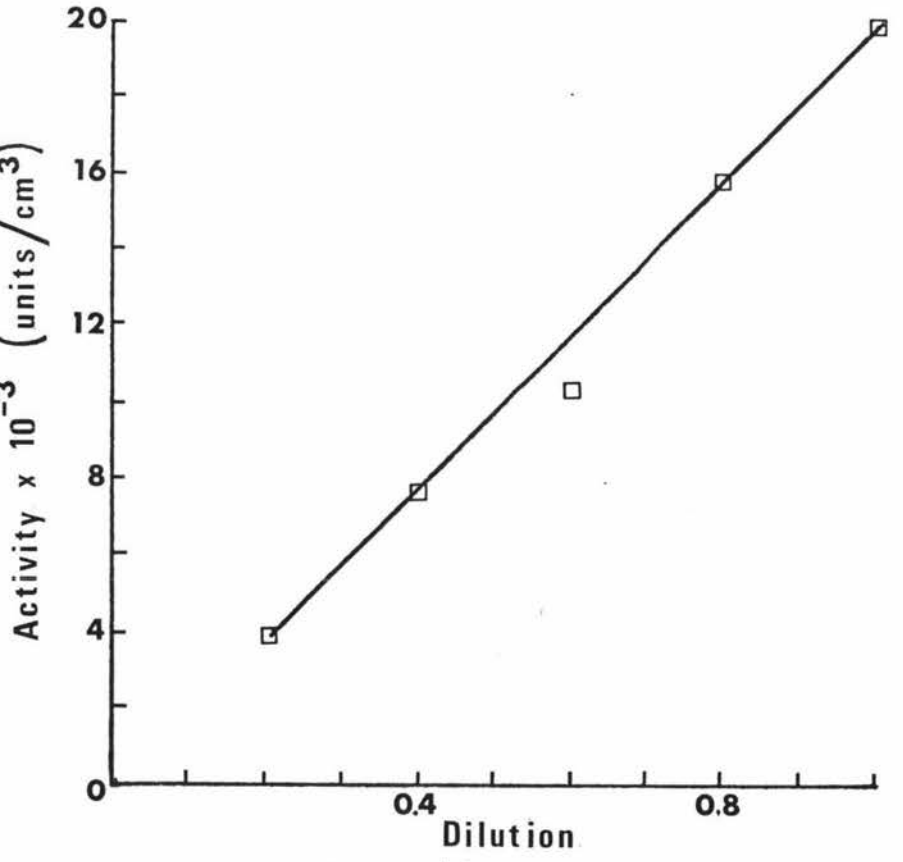

Fig. 10 Antibacterial activity of a neutralized, 0.2M citrate, pH 2.0, pancreas extract at various dilutions

4. Isolation and Purification of Antibacterial Factors from Bovine Seminal Plasma and Pancreas

4.1 Extraction with Deoxyribonucleic Acid

Initially, six attempts were made to isolate antibacterial activity from BSP using deoxyribonucleic acid to adsorb the active fraction following Schollum's procedure (1977) (Methods 2.1). For each preparation, the various fractions were analyzed, and the per cent units adsorbed to DNA and subsequently recovered from DNA were determined. For example:

	total units:
initial BSP	2,311,500
supernatant 1	606,900
NaCl wash 1	168,000
NaCl wash 2	38,400
NaCl wash 3	not detected
ppt 1:7	30,000

units adsorbed to DNA* : 1,498,200

% of initial activity adsorbed to DNA : 1,498,200/2,311,500 = 64.8%

% of adsorbed activity in ppt 1:7 : 30,000/1,498,200 = 2.0%

% of initial activity in ppt 1:7 : 30,000/2,311,500 = 1.3%

The per cent units adsorbed and recovered for each preparation are presented in Table V. Although good adsorption of activity with DNA was obtained (59%), the average recovery of activity from the DNA was low, about 5%. The recovery in the 1:7 precipitate based on total, initial BSP units was only 3%, and the specific activity was low (500 units/mg), as determined for preparations V and VI.

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* determined by difference : total initial activity less activity present in supernatant 1 and NaCl washes.

Table V. Deoxyribonucleic acid extraction of antibacterial activity from six batches of BSP

Preparation	% of Initial Activity Adsorbed to DNA	% of Adsorbed Activity in PPT. 1:7	% of Initial Activity in PPT. 1:7	% of Initial Activity Recovered in All Fractions
I	64.8	2.0	1.3	36.5
II	72.5	0.6	0.4	27.9
III	84.2	2.3	1.9	17.7
IV	72.6	0.9	0.6	27.4
V	26.5	15.8	6.0	58.1
VI	47.2	11.1	5.3	79.4
average	59.4	5.4	2.6	41.2

An attempt was made to recover more activity in preparation II. The 1:1 supernatant was concentrated 100 times and analyzed, but no activity was detected. Next, the DNA was redissolved in water, retreated with citrate and reprecipitated with ethanol. An aliquot of the supernatant was concentrated and analyzed, but no activity was detected. Ethanol was added to the remainder of the supernatant to give a 1:3 precipitate; this was assayed but no activity was detected.

Failure to account for all of the initial activity could be due: i) to material which remained bound to the DNA or ii) to material present in precipitates 1:1 and 1:3 which was not detected at the concentrations (5 and 10 mg ppt/cm^3) assayed.

The best recovery was obtained in runs V and VI in which a more gentle treatment of DNA was employed, and in which the citrate was allowed to stand with the DNA for a longer time before the final dilution was made.

Both calf thymus DNA (runs I, II, IV, V) and salmon DNA (runs III, VI) were used with no significant differences in recovery.

4.2 Extraction with Cellulose Phosphate

In response to the low recovery of purified antibacterial substance obtained with the DNA extraction procedure, a second isolation procedure was developed. Cellulose phosphate seemed a logical alternative to DNA, as a negatively charged substance to which the basic, antibacterial substance might adsorb.

4.2.1 Extraction of Antibacterial Factor(s) from Bovine Seminal Plasma

A preliminary study (Methods 2.3.2.1.1) on the adsorption of BSP active material to cellulose phosphate, over intervals up to one hour,

showed that 91% of the original activity was adsorbed after 5 min. A 15 min adsorption period was chosen for all succeeding cellulose phosphate treatments.

With regard to release of the active fraction from cellulose phosphate following citrate treatment, an interesting observation was made in this, and succeeding experiments. Thus, in 43 cellulose phosphate treatments of BSP, an average recovery of 174% was obtained in the various fractions. That is, the total activity recovered was greater than the initial activity added to the cellulose phosphate. The recovery ranged from 77 to 269%, but was greater than 100% in all but two treatments.

4.2.1.1 Ratio of Cellulose Phosphate to BSP

The recovery of activity associated with different ratios of cellulose phosphate to bovine seminal plasma (Methods 2.3.2.1.2.i and ii) is presented in Table VI.

In the second experiment the mixtures were brought to a constant volume of 5.5 cm^3 with buffer. However, as percentages were calculated on total activity, both experiments were directly comparable. The results indicate that only about 20% of the activity was left in the first supernatant, when the CP to BSP ratio was 1.2, whereas 80% was left when the ratio was 0.1. Consequently, the per cent recovered units in the final eluate was much greater with a ratio of 1.2 (70%) and decreased as the ratio decreased. There was little difference in the specific activities of the final eluates, although the 1.2 ratio yielded a preparation with a slightly lower specific activity.

An additional experiment (Methods 2.3.2.1.2.iv) relating to the adsorption of antibacterial material to cellulose phosphate, involved the treatment of BSP with low amounts of cellulose phosphate, followed by retreatment of the first supernatnant with a greater amount of cellulose phosphate (Table VII).

Table VI. Recovery of activity following changes in the cellulose phosphate to BSP ratio

	CP Slurry (cm^3)	BSP (cm^3)	Ratio of CP:BSP	% of Recovered Activity in 1st supernatant	% of Recovered Activity in final eluate	Specific Activity (units/mg)
I.	0.5	5.0	0.1	76	18	
	0.5	2.5	0.2	50	44	
	1.0	2.5	0.4	31	63	
	1.0	1.25	0.8	15	79	
	1.5	1.25	1.2	11	84	
II.	0.25	2.5	0.1	81	15	
	0.5	2.5	0.2	69	28	1450
	1.0	2.5	0.4	54	42	1300
	2.0	2.5	0.8	32	62	1400
	3.0	2.5	1.2	33	60	1120

Table VII. Cellulose phosphate treatment of first supernatants obtained following the treatment of BSP with low amounts of cellulose phosphate

	Treatment	% of Initial Activity Adsorbed to CP*	% of 1st Supernatant Activity Adsorbed to CP	% of Adsorbed Activity Eluted from CP
1.	0.25 cm^3 CP + 2.5 cm^3 BSP,	13		115
	3.0 cm^3 CP retreatment		77	82
2.	0.5 cm^3 CP + 2.5 cm^3 BSP,	29		112
	3.0 cm^3 retreatment		87	74

* determined by difference : initial observed activity less activity in first supernatant.

Approximately 80 to 90% of the activity left in the first supernatant, following the initial cellulose phosphate treatment of BSP, was adsorbed to cellulose phosphate in a second treatment and subsequently eluted with citrate.

4.2.1.2 Effect of Heat on the Adsorption of Antibacterial Factor(s) to Cellulose Phosphate (Methods 2.3.2.1.3)

In both heated and unheated mixtures of cellulose phosphate and BSP, 11% of the recovered activity was left in the first supernatant and buffer wash, and 89% of the activity occurred in the final supernatant. The specific activities of the final supernatants from the heated and unheated samples were 3700 and 3500 units/mg respectively. Thus, heat treatment (60^{o}, 1 h) of BSP in the presence of cellulose phosphate seemed to have no beneficial effects in the purification process and was not used.

4.2.1.3 Effect of Varying the pH of the Cellulose Phosphate Slurry (Methods 2.3.2.1.4)

The effects of pH variation on the adsorption and release of active material from cellulose phosphate are presented in Table VIII.

Table VIII. The effect of pH on the adsorption and release of BSP activity from cellulose phosphate

Cellulose Phosphate pH	% of Recovered Activity In: 1st Supernatant + Wash	Final Eluate	Specific Activity of Final Eluate (units/mg)
5.8	45	55	2610
7.4	55	45	2740
8.0	70	30	1740

The specific activity of the final eluate following treatment at pH 8.0 was 65% of that obtained for the final eluates after pH 5.8 and 7.4 treatments. The differences in recoveries and specific activities following treatment at pH 5.8 and 7.4 were not significant, and the continued use of cellulose phosphate equilibrated at pH 7.4 was justified.

4.2.1.4 Elution of Proteins Bound to Cellulose Phosphate

4.2.1.4.1 Elution with Sodium Citrate (Methods 2.3.2.1.6.i)

Initially, 0.5 cm^3 of 1.6M sodium citrate was used to elute the active material from the cellulose phosphate. For comparison, three, 3.0 cm^3 CP pellets with adsorbed activity were treated with 0.5 cm^3 of 1.6M sodium citrate, 1.5 cm^3 of 1.6M citrate and 1.0 cm^3 of saturated citrate (22^o) respectively. The results demonstrated that a 1.0 cm^3 saturated citrate wash resulted in a greater release of activity from the cellulose phosphate (92%) than did a 0.5 cm^3 1.6M citrate wash (50%). However, an equally good release was obtained with 1.5 cm^3 of 1.6M citrate, which yielded a final eluate with a specific activity (3420 units/mg) 45% higher than the final eluate (1880 units/mg) from the saturated citrate treatment. That is, the saturated citrate is presumed to have removed more contaminating protein than did the 1.6M citrate. The specific activity of the final eluate from the 1.5 cm^3 wash was 68% higher than that from the 0.5 cm^3 wash (1110 units/mg), because more activity was eluated with the former treatment.

A further experiment confirmed the inefficient removal of active material from a 3.0 cm^3 CP pellet with a 0.5 cm^3 1.6M citrate wash. The results in Table IX indicate that active material, still bound to the cellulose phosphate following the citrate wash, could be eluted with additional citrate washes.

Table IX. Elution of antibacterial activity from cellulose phosphate following repeated citrate washes

0.5 cm^3 1.6M Citrate Wash	% of Recovered Activity in Citrate Eluates	Specific Activity (units/mg)	Protein in Citrate Eluates (mg)
1	39	1090	23.2
2	7	250	19.3
3	20	3620	3.5

The specific activity of the third citrate wash was the greatest, which indicated that a wash, which would remove mainly contaminating protein but little active protein, might be a useful step in the purification procedure.

Based on the results from these two experiments, a 1.5 cm^3 1.6M sodium citrate wash was used in succeeding treatments of a 3.0 cm^3 CP pellet.

4.2.1.4.2 Elution with Sodium Chloride

i. Variation of NaCl Concentration

The use of NaCl to remove contaminating protein from cellulose phosphate adsorbed with BSP was studied. Two experiments compared the effects of various concentrations of NaCl on the removal of protein and activity from the cellulose phosphate (Methods 2.3.2.1.6.ii.a). The results of the first experiment (Table XA) demonstrate that the specific activity of the final eluate following a 0.2M NaCl wash (3500 units/mg) was greater than that following a water wash (2700 units/mg). Although 0.75M and 1.0M NaCl washes also removed contaminating protein, approximately 70% of the recovered activity was washed from the cellulose phosphate

Table X. Elution of proteins from cellulose phosphate with different sodium chloride concentrations

	NaCl	% of Recovered Activity in:			NaCl Wash:		Specific Activity
		First	NaCl	Final	Protein	S.A.	of Final Eluate
	(M)	Supernatant	Wash	Eluate	(mg)	(units/mg)	(units/mg)
XA.	0	11	ND*	84	3.8	-	2730
	0.25	11	2	83	4.8	200	3470
	0.75	9	67	18	14.4	1830	3000
	1.0	7	75	12	14.9	2340	2560
XB.	0.25	11	ND	85	6.5	-	1490
	0.4	11	21	68	10.1	400	1530
	0.6	7	41	51	12.9	800	2540
	0.75	6	60	33	17.5	1000	2530

* ND = no activity detected.

as well. Consequently, the specific activities of the final eluates were lower than the specific activity for the final eluate obtained following a 0.2M wash.

In the second experiment, the use of NaCl concentrations between 0.25M and 0.75M was considered. The results, presented in Table XB, indicate that activity was sacrificed if a concentration greater than 0.25M NaCl was used. Although the specific activity of the final eluate was not enhanced with a 0.4M wash, an increase in the specific activities of the final eluates following 0.6M and 0.75M washes was obtained. However, the 50 to 70% loss of activity which occurred with the latter washes did not warrant their use.

Fig. 11 illustrates the elution of antibacterial activity from cellulose phosphate with different NaCl concentrations.

ii. Variation of NaCl Volume

The volume of the NaCl wash was considered next (Methods 2.3.2.1. 6.ii.b). The results obtained following one, 7.5 cm^3 wash of a 3.0 cm^3 CP pellet were compared with those after three, 2.5 cm^3 washes. First, 0.5M NaCl was used to ensure detectable activity in each wash. Then 0.2M NaCl was used for comparison. The results (Table XI) indicate that one wash with a volume equal to the total volume of three washes removed an equivalent amount of activity from the cellulose phosphate pellet.

The specific activities of the final eluates following 0.2M washes were 43% greater than those following 0.5M washes, due to a lower loss of activity with the former treatment.

Next, the results obtained following one, 7.5 cm^3 wash of a 3.0 cm^3 pellet were compared with those following two or six washes with a total volume of 15.0 cm^3. As no activity was detected in the 0.2M NaCl washes, a comparison of the amount of protein which was removed is presented in Table XII. Again there was little difference between the results obtained

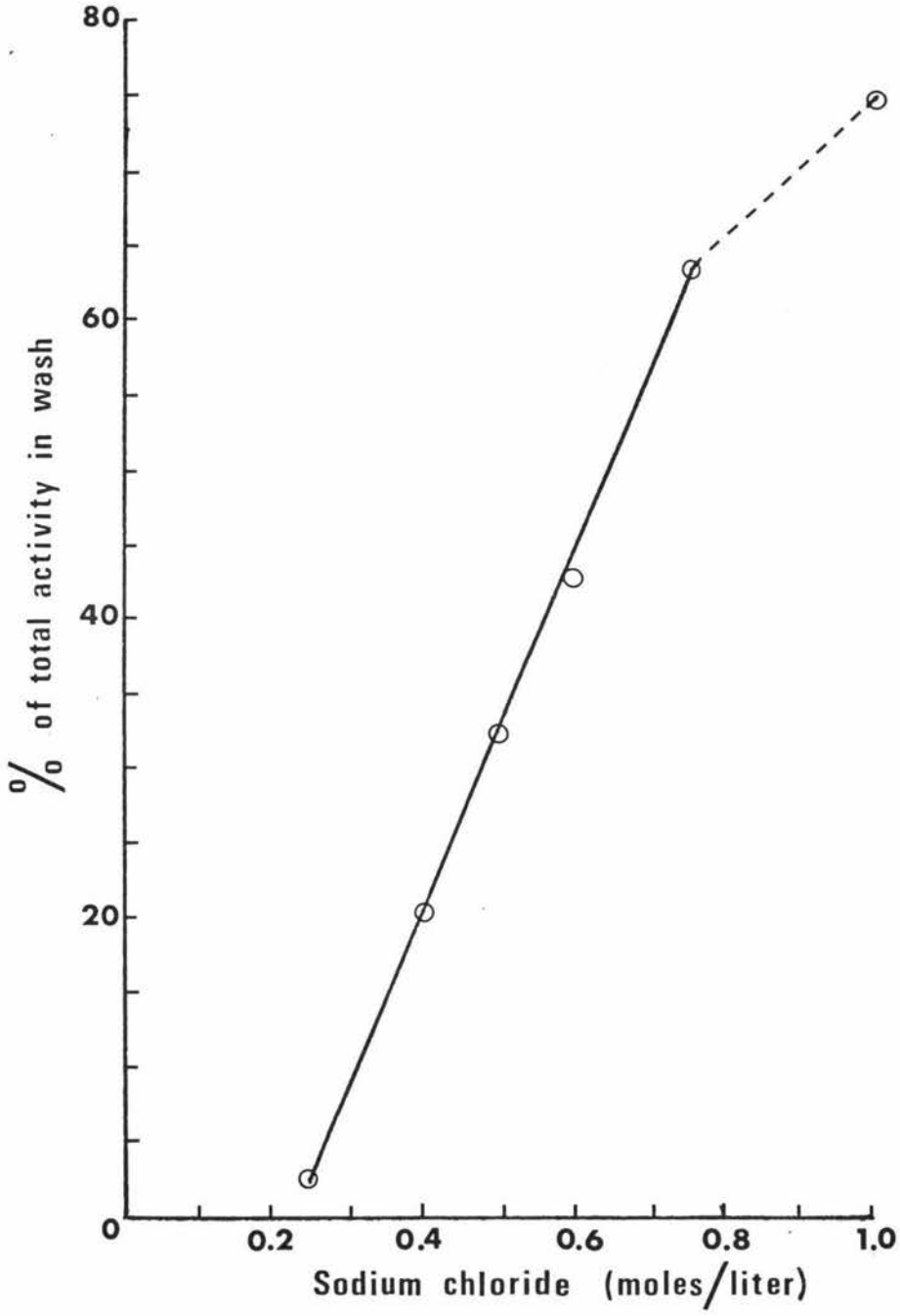


Fig. 11 Elution of BSP antibacterial activity from cellulose phosphate with sodium chloride

Table XI. Comparison of elution of BSP activity from CP with one, 7.5 cm^3 NaCl wash or three, 2.5 cm^3 NaCl washes

% Recovered Activity In:	0.5M NaCl		0.2M NaCl	
	one wash	three washes	one wash	three washes
first supernatant	10	10	13	14
NaCl wash 1	53	13	6	1.5
NaCl wash 2		18 (50)		2.2 (6)
NaCl wash 3		19		2.1
final eluate	33	35	79	76
Specific Activity of Final Eluate (units/mg)	1300	1200	2400	2000
Protein in NaCl Washes (mg)	42	53	35	32

Table XII. Comparison of elution of BSP protein from CP with different volumes and numbers of NaCl washes; and final washes of sodium chloride or sodium citrate

NaCl Washes (Final Wash)	Protein (mg) in NaCl Washes						Total Protein in NaCl Washes (mg)	Specific Activity of Final Eluate (units/mg)
	1	2	3	4	5	6		
1 x 7.5 cm^3 NaCl (7.5 cm^3 1.0M NaCl)	22.3						22.3	3290
2 x 7.5 cm^3 NaCl (1.5 cm^3 1.6M citrate)	26.2	7.6					33.8	3430
6 x 2.5 cm^3 NaCl (1.5 cm^3 1.6M citrate)	19.0	6.0	3.6	2.2	1.1	1.1	33.0	3500

following two or six washes with a total volume of 15.0 cm^3. As expected, less protein was removed when half the wash volume was used.

This experiment also compared the use of a final 7.5 cm^3 1.0M NaCl wash with a 1.5 cm^3 1.6M sodium citrate wash to elute the activity from the cellulose phosphate. Each wash released 88 to 91% of the recovered activity, and the final specific activities obtained were slightly greater following the 15.0 cm^3 NaCl washes.

A comparison between the number of NaCl washes and the total activity recovered is presented in Table XIII. The results indicate that greater activity was recovered with fewer NaCl washes. Perhaps the NaCl elutes inhibitory proteins which mask the activity of the antibacterial substance, which is also eluted from the cellulose phosphate with NaCl. With fewer washes, the greater volumes of each wash may provide a dilution effect. Thus, the masking of active protein by other proteins would be less pronounced, and consequently a greater recovery would be observed.

The importance of wash volumes with respect to the size of the cellulose phosphate pellet was further exemplified in the experiment in which 3.0 cm^3 of cellulose phosphate was washed as one pellet, or divided and washed as three, 1.0 cm^3 pellets (Methods 2.3.2.1.2.iii). The results (Table XIV) illustrate that more activity was recovered from the cellulose phosphate which was divided and then washed. That is, the smaller CP pellets were more effectively washed. Consequently, the specific activity of the final eluate was greater (75%).

However, the total wash volumes for the divided pellet were three times greater than for the undivided pellet. Thus, one may argue that if the wash volumes were kept constant, the results from each would approximate each other. Perhaps, yet the experiment does illustrate that a sufficient wash volume must be used to achieve maximum recovery and specific activity.

Table XIII. Comparison of recovery of BSP activity from CP with different numbers of NaCl washes

Experiment	No. of Washes	Volume of Washes (cm^3)	Total Wash Volume (cm^3)	% Activity Recovered
I	1	7.5	7.5	235
	2	7.5	15.0	198
	6	2.5	15.0	176
II	1	7.5	7.5	179
	3	2.5	7.5	144
III	1	7.5	7.5	154
	3	2.5	7.5	123

Table XIV. Comparison of elution of BSP activity from a 3.0 cm^3 CP slurry washed as a single pellet or as three, 1.0 cm^3 pellets

Total Units in:	3.0 cm^3 Pellet	3.0 cm^3 $\rightarrow$ 3 x 1.0 cm^3 Pellets
first supernatant	6105	8745
buffer wash 1	1560	3074
0.5M NaCl wash	9130	34,200
buffer wash 2	1541	1595
final eluate	16,330	59,280
Specific Activity of Final Eluate (units/mg)	930	3700
% Activity Recovered	77	182

Similarly, the same point was illustrated when the wash volumes were kept constant and the CP slurry was varied. The specific activity of the final eluate obtained from a 1.0 cm^3 pellet (2000 units/mg) was greater than that from a 3.0 cm^3 pellet (930 units/mg) treated identically.

4.2.1.4.3 Elution with Sodium Phosphate

The effectiveness of 0.1 to 0.5M sodium phosphate buffer, pH 7.8, 1.0M sodium chloride and 1.6M sodium citrate in the elution of antibacterial activity from cellulose phosphate was compared (Methods 2.3.2.1.6.iii). The results (Table XV) illustrate that the antibacterial factor was eluted less readily with sodium phosphate than with sodium chloride. No activity was detected in the final eluates following treatment with sodium phosphate of comparable ionic strength to sodium chloride, which yielded 43% of the citrate figure. The greater ionic strength of 1.6M sodium citrate gave excellent recovery and a final eluate with a higher specific activity.

Table XV. Relative effectiveness of sodium phosphate and sodium chloride as eluates compared to 1.6M sodium citrate

	0.1M Sodium Phosphate Buffer	0.25M Sodium Phosphate Buffer	0.4M Sodium Phosphate Buffer	0.5M Sodium Phosphate Buffer	1.0M Sodium Chloride	1.6M Sodium Citrate
Ionic strength (μ)	0.6	1.5	2.4	3.0	1.0	9.6
Relative Recovery in Eluate	ND*	ND*	28	43	43	100
Specific Activity of Eluate (units/mg)	-	-	290	430	420	3420

* ND = no activity detected.

4.2.2 Extraction of Antibacterial Factor(s) from Pancreas Extracts

A preliminary study (Methods 2.3.2.2.1) on the adsorption of pancreas antibacterial activity to cellulose phosphate, over intervals of up to one hour, showed that 95% of the original activity was adsorbed after 5 min. A 15 min adsorption period was chosen for all succeeding cellulose phosphate treatments.

Citrate treatment resulted in the elution of 88% of the activity adsorbed to the cellulose phosphate; this was 84% of the initial activity used.

Unlike CP treatment of BSP, no increase in activity over the initial units treated was observed. For nine treatments, an average recovery of 77% was obtained.

4.2.2.1 Ratio of Pancreas Extract to Cellulose Phosphate (Methods 2.3.2.2.1)

The treatment of 2.5 cm^3 pancreas extract, bovine seminal plasma and later, acid-citrate-heat treated bovine seminal plasma (T-BSP, see Methods 2.3.2.1.5) with various volumes of cellulose phosphate slurry were compared. The results are summarized in Table XVI.

A comparison between pancreas and BSP showed that less activity was left in the first supernatant following a 0.5 cm^3 CP treatment of the pancreas extract; that is, 31% for pancreas as compared to 56% for BSP. The greater adsorption of pancreas activity to CP may be a reflection of the higher initial specific activity of the pancreas extract, 1530 units/mg as compared to 320 units/mg for BSP. Consequently, the final pancreas eluate, after a 0.5 cm^3 CP treatment, contained a greater percentage of the recovered activity (69%) than did the equivalently treated BSP (44%).

The final eluates obtained following a 0.5 cm^3 CP treatment had

Table XVI. Comparison of elution of activity from CP treated with pancreas extract, BSP or acid - citrate - heat treated BSP (T-BSP)

Sample	Cellulose Phosphate Slurry Volume (cm^3) 0.5	1.0	2.0	3.0
	% of Recovered Activity in First Supernatant			
Pancreas Extract	31	24	15	7
BSP	56	38	15	9
T-BSP	61	48	37	19
	% of Recovered Activity in Final Eluate			
Pancreas Extract	69	76	85	93
BSP	44	62	85	91
T-BSP	39	52	63	81
	S.A of Final Eluate (units/mg) (Purification of Initial Sample)			
Pancreas Extract	21,250 (14x)	16,500 (11x)	11,700 (8x)	10,980 (7x)
BSP	6270 (20x)	4690 (15x)	4500 (14x)	3970 (13x)
T-BSP	2200 (2x)	1390 (1.3x)	1400 (1.3x)	1540 (1.5x)

higher specific activities than those obtained following a 3.0 cm^3 CP treatment. These increases were 48% and 37% for pancreas and BSP respectively. A consideration of these results led to the use of a ratio of 0.5 cm^3 CP slurry to 2.5 cm^3 pancreas extract for routine preparations of pancreas factor.

The specific activities of pancreas final eluates were three times higher than those obtained for BSP final eluates. These results led to the consideration of the effects of a comparable acid-citrate-heat treatment of BSP on the specific activities of the final eluates following CP treatment. Although a 3.3 fold purification over BSP was achieved with the treatment, an increase in specific activity comparable to that obtained for pancreas extract following CP treatment was not observed. In fact, the specific activities obtained were three times less than those obtained for untreated BSP, and ten times less than the specific activities of final pancreas eluates.

4.2.3 Extraction of Antibacterial Factor(s) from Human Seminal Plasma (HSP)

No detectable activity was present in the first supernatant following treatment of HSP with cellulose phosphate (i.e. 100% adsorption to CP). Citrate treatment resulted in the elution of 58% of the adsorbed activity. The specific activity increased from 450 units/mg (HSP) to 1470 units/mg (treated HSP), a 3.3 fold purification. The comparison between the antibacterial activity of human seminal plasma and the activities in bovine pancreas and seminal plasma was not continued beyond this preliminary experiment.

4.3 Ethanol Precipitation of the Antibacterial Factors

4.3.1 Bovine Seminal Plasma Eluate from Cellulose Phosphate (Methods 2.4.1)

The ethanol precipitation profiles at 0^{o} for two cellulose phosphate eluates, at 3.8 (I) and 2.3 (II) mg protein/cm^3, respectively, are presented in Table XVII. The increase in specific activities observed for the 1:7 precipitates represented 3.6 and 2.1 fold purifications over the final CP eluates.

The ethanol precipitations carried out during the DNA isolation procedure were done at room temperature. Therefore, a comparison of the ethanol precipitation profile of a CP eluate (4.5 mg/cm^3, 2090 units/mg) at 0^{o} and 25^{o} was made. The results (Table XVIII) demonstrate that temperature variations cause major differences in the pattern of precipitation.

At 0^{o}, 80% of the protein was precipitated with the 1:1.5 ethanol cut; this resulted in all successive cuts having high specific activities. At 25^{o}, however, contaminating protein appeared to precipitate more gradually, and therefore, only the 1:7 precipitate had a specific activity comparable to that obtained in precipitates 1:2.4 to 1:7 at 0^{o}. Also, whereas 63% of the original activity was recovered at 0^{o}, only 42% was recovered at 25^{o}.

Ethanol precipitation of the same CP eluate was studied more closely at 0^{o}. The precipitation profiles of two experiments (I, II) are presented in Table XIX. The results demonstrate that the material with the highest specific activity is precipitated between the 1:2 and 1:4 ethanol cuts.

All preceeding ethanol precipitations were carried out on final cellulose phosphate eluates diluted with distilled water. However,

Table XVII. Ethanol precipitation profiles of two BSP - CP eluates at 0^{o}

Ratio of CP Eluate to Ethanol (v/v)	% of Recovered Activity		% of Recovered Protein		Specific Activity (units/mg)	
	I	II	I	II	I	II
ppt 1:1	ND*	ND*	51	26	-	-
ppt 1:3	62	93	34	71	2590	2750
ppt 1:7	30	4	6	2	6880	4200
final supernatant	1	ND*	9	1		

I. CP eluate : 3.8 mg protein/cm^3.
II. CP eluate : 2.3 mg protein/cm^3.
* ND = no activity detected.

Table XVIII. Comparison of ethanol precipitation of a BSP - CP eluate at 0^{o} and 25^{o}

Ratio of CP Eluate to Ethanol (v/v)	% of Recovered Activity		% of Recovered Protein		Specific Activity (units/mg)	
	0^{o}	25^{o}	0^{o}	25^{o}	0^{o}	25^{o}
ppt 1:1.5	30	ND*	80	24	590	-
ppt 1:2.2	-	9	-	41	-	290
ppt 1:2.4	32	-	10	-	5200	-
ppt 1:2.5	-	0.9	-	1	-	1110
ppt 1:3.5	19	41	5	20	6110	2770
ppt 1:7	19	49	4	12	7030	5310
final supernatant	1.5	0.3	0.3	3		

* ND = no activity detected.

Table XIX. Ethanol precipitation profiles of two BSP - CP eluates at 0^{o}

Ratio of CP Eluate to Ethanol (v/v)	% of Recovered Activity	% of Recovered Protein	Specific Activity (units/mg)
I. ppt 1:1	2	32	140
ppt 1:1.5	23	40	1210
ppt 1:2	7	6	2600
ppt 1:7	68	22	6620
final supernatant	ND*	ND*	-
II. ppt 1:2	66	89	1880
ppt 1:4	32	9	8570
ppt 1:5.5	2	1	3800
ppt 1:7	0.2	ND*	1850
final supernatant	ND*	ND*	-

* ND = no activity detected.

for reproducible results the pH must be controlled during protein precipitation (Green and Hughes, 1967). The ethanol precipitation of a final CP eluate diluted with 0.01M sodium phosphate buffer, pH 7.4, (4.2 mg/cm^3, 4400 units/mg) was compared with that of an eluate diluted with distilled water (3.3 mg/cm^3, 6440 units/mg).

Although the precipitation profiles of each sample (Table XX) were quite similar, final eluates with higher specific activities were obtained under buffered conditions. The results also indicate that a finer ethanol cut, that is, between 1:2 and 1:4, yields preparations with even greater specific activities.

4.3.1.1 Criteria of Purity of BSP Preparations

4.3.1.1.1 Native Polyacrylamide Gel Electrophoresis

Native gel electrophoresis was used very briefly for examining the number of protein components of a sample. For example, the native gel profile for whole bovine seminal plasma is presented in Fig. 12. In comparison to the five major components of BSP, gel electrophoresis of a BSP precipitate 1:7 (6880 units/mg) showed a major band near the gel's origin and a fainter band about two-thirds of the way down the gel. Such results were a visible indication of the effectiveness of the purification procedure.

4.3.1.1.2 SDS Gel Electrophoresis

Both specific activity and SDS gel electrophoresis were used routinely throughout the study for monitoring the relative purity of various preparations. For example, Fig. 13 shows the SDS gel scans of 8 to 60 µg samples of a 1:4 precipitate with a specific activity of 11,625 units/mg. As the amount of sample was increased the asymmetry of the peak increased, and the presence of a larger molecular weight

Table XX. Comparison of ethanol precipitation of buffered and unbuffered BSP - CP eluates

Ratio of CP Eluate to Ethanol (v/v)	% of Recovered Activity		% of Recovered Protein		Specific Activity (units/mg)	
	buffered	unbuffered	buffered	unbuffered	buffered	unbuffered
ppt 1:2	70	75	89	90	4170	2990
ppt 1:3	10	5	4	2	13,220	9500
ppt 1:4	18	15	5	5	21,430	11,620
final supernatant	2	5	2	3	4100	6500

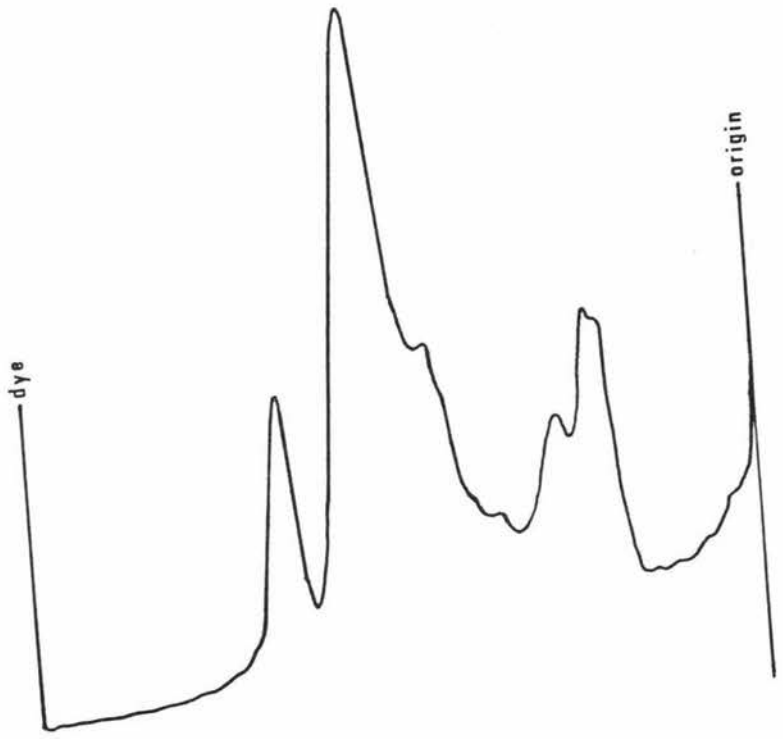


Fig. 12 Native polyacrylamide gel electrophoresis of whole bovine seminal plasma.

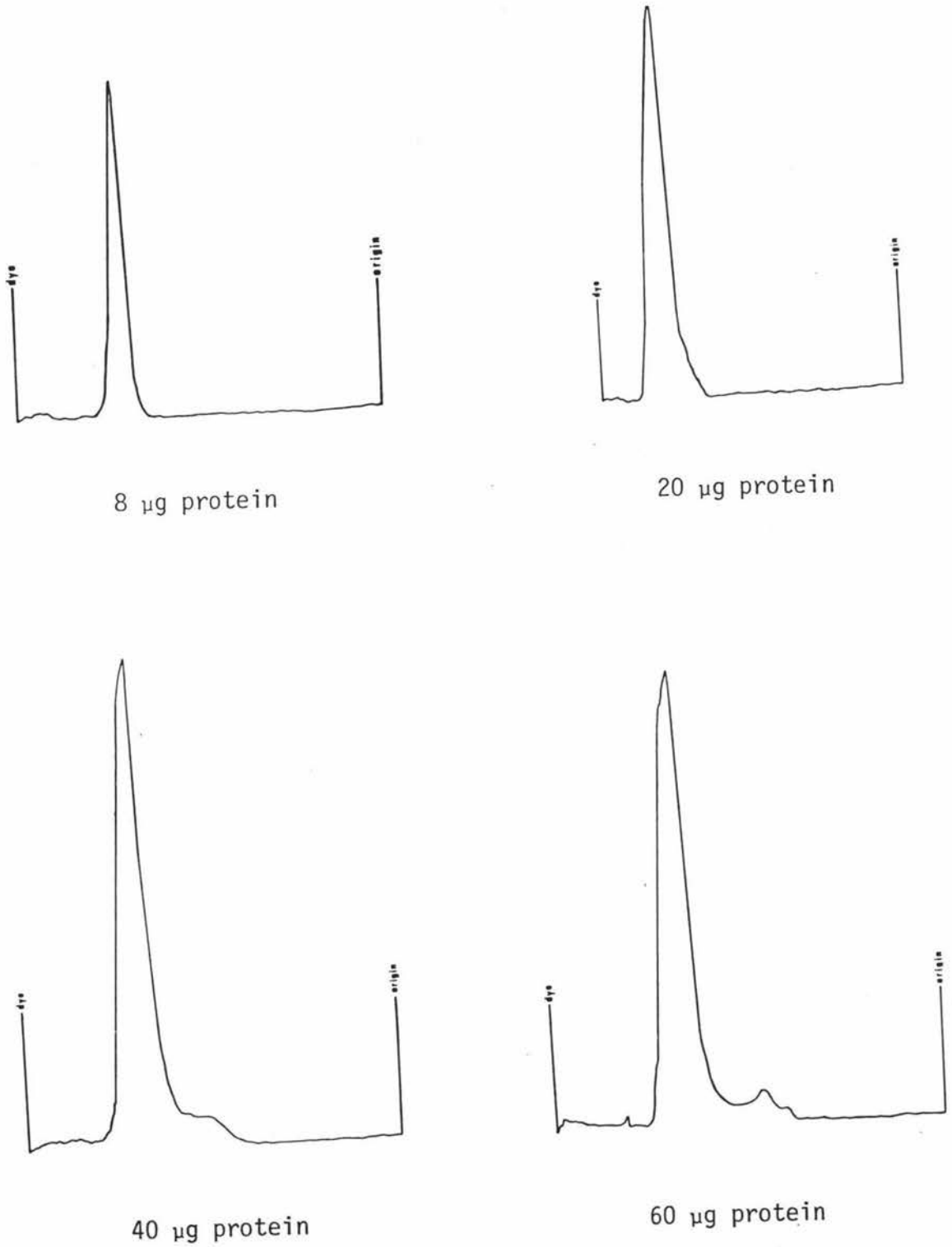


Fig. 13 SDS gel electrophoresis of different amounts of a 1:4 BSP precipitate (11,620 units/mg protein).

contaminant was observed.

The SDS gel scans of BSP ppt 1:4[1] (17,370 units/mg), which was used to immunize rabbit A, and BSP ppt 1:3[1] (16,860 units/mg), which was used to immunize rabbit B, are presented in Fig. 14. At the sample amount used (5 µg), both preparations appeared to contain only one component. The amino acid analysis was done on the ppt 1:3[1] before it was concentrated by lyophilization (18,400 units/mg).

The average relative mobility (6 determinations, 4 samples) of the BSP antibacterial factor as compared to the mobility of lysozyme, which was arbitrarily set at 1.0, was 1.06.

4.3.1.2 Reprecipitation of a BSP 1:4 precipitate

Reprecipitation of an aqueous solution of a 1:4 precipitate yielded even better preparations with higher specific activities. The results, summarized in Table XXI, show the increase in specific activity which was obtained following the reprecipitation of two 1:4 solutions with specific activities of 9960 and 8470 units/mg respectively. The reprecipitations resulted in a loss of only 17% and 8% of the activity respectively.

4.3.2 Pancreas Eluate from Cellulose Phosphate

As the specific activities of the pancreas extracts were three times greater than those of BSP, a direct ethanol precipitation of the pancreas extract was attempted. The results (Table XXII) show that the greatest per cent of the active protein precipitated in the 1:1.5 ethanol cut. The specific activity obtained, however, was very low compared to that which had been obtained following CP treatment of the pancreas extract (21,000 units/mg). The direct precipitation of the pancreas extract resulted in the precipitation of active protein across the range of ethanol cuts used, and was consequently of no benefit in the purification process.

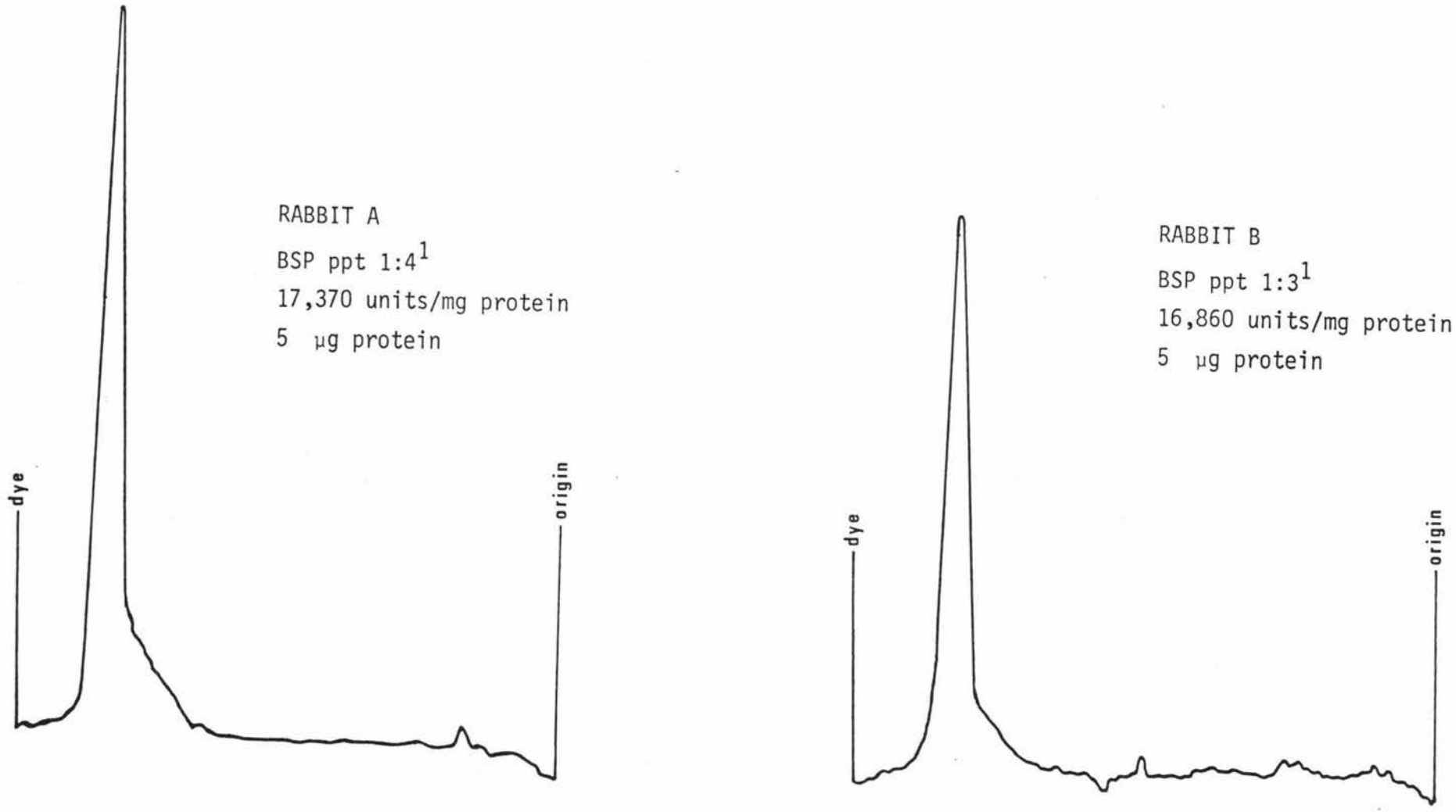


Fig. 14 SDS gel electrophoresis of BSP preparations used to immunize rabbits A and B.

Table XXI. Ethanol reprecipitation of two aqueous solutions of BSP, 1:4 ethanol precipitates

Ratio of 1:4 ppt. Solutions to Ethanol (v/v)	% of Recovered Activity	% of Recovered Protein	Specific Activity (units/mg)
I. ppt 1:2[1]	7	19	4720
ppt 1:4[1]	57	39	17,370
ppt 1:7[1]	34	29	13,930
II. ppt 1:2[1]	87	91	11,380
ppt 1:3[1]	12	8	18,400
ppt 1:5[1]	0.8	0.7	26,820

I 9960 units/mg protein.
II 8470 units/mg protein.

Table XXII. Ethanol precipitation profile of a crude pancreas extract

Ratio of Pancreas Extract to Ethanol (v/v)	% of Recovered Activity	% of Recovered Protein	Specific Activity (units/mg)
ppt 1:1.5	52	44	2690
ppt 1:2	17	16	2380
ppt 1:2.5	12	11	2570
ppt 1:3.5	4	5	1850
ppt 1:4	2	3	2060
ppt 1:5.5	4	5	1650
ppt 1:7	3	3	2260
final supernatant	5	14	850

The ethanol precipitation profile of a final pancreas-CP eluate, with 0.3 mg protein/cm^3 and 16,500 units/mg, is presented in Table XXIII. Unlike the BSP factor, the active pancreas material precipitated very early in the profile. The majority of the activity occurred in fraction 1:1.5. These early fractions contain high amounts of citrate, which also precipitates in the early stages of ethanol precipitation. SDS gel electrophoresis of (i) 13 μg of the 1:1.5 precipitate and (ii) 9 μg of the 1:2.5 precipitate, indicated that both fractions were composed of two components with very similar mobilities.

4.3.2.1 Effect of pH on the Ethanol Precipitation of Pancreas Material

In an effort to separate the two components, ethanol precipitations were carried out at three different pH values: 5.8, 7.0 and 8.0 (Methods 2.4.2). The comparison is presented in Table XXIV.

A comparison of the scans of the 1:2 precipitates following SDS gel electrophoresis is presented in Fig. 15. The relative mobilities of each peak with respect to lysozyme (mobility arbitrarily set at 1.0) were as follows:

	$peak_1$	$peak_2$	
pH 5.8	0.88	2.11	
pH 7.0	0.98	1.24	5 μg sample (one peak) 1.05
pH 8.0	1.09	1.24	

Although the two components were not separated at any of the pH values tested, the best preparation with regard to total units and specific activity was precipitate 1:2 at pH 7.0. All succeeding ethanol precipitations were carried out with the CP final eluate diluted with 0.01M sodium phosphate buffer, at pH 7.4, as used for BSP-CP eluates.

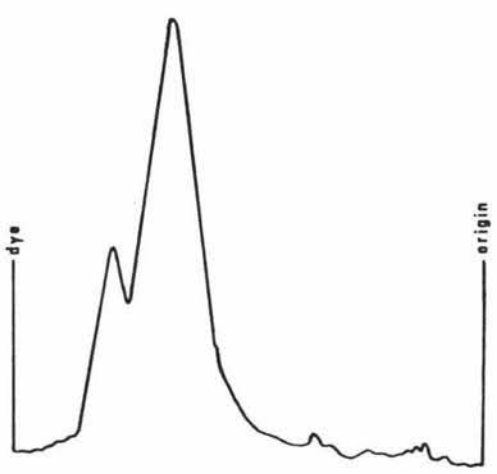
Table XXIII. Ethanol precipitation profile of a pancreas-CP eluate

Ratio of CP-Eluate to Ethanol (v/v)	% of Recovered Activity	% of Recovered Protein	Specific Activity (units/mg)
ppt 1:1.5	84	83	14,350
ppt 1:2.5	14	13	16,000
ppt 1:4	1	3	6230
ppt 1:7	ND*	0.9	-

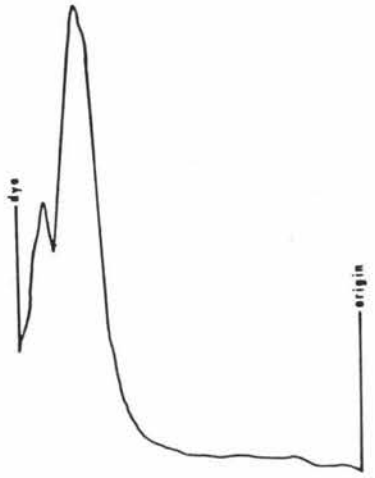
* ND = no activity detected.

Table XXIV. Effect of pH on the ethanol precipitation of a pancreas-CP eluate

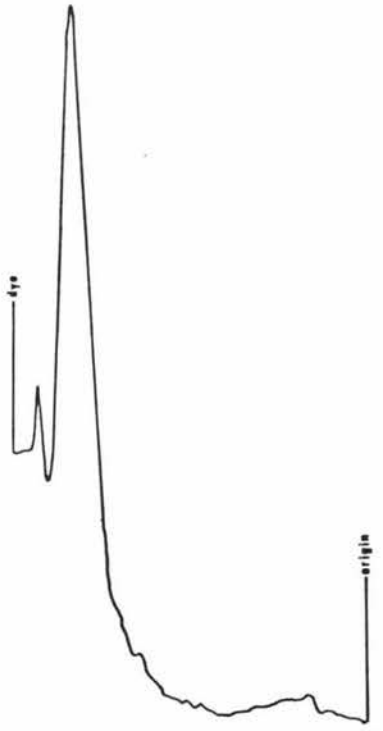
Ratio of CP-Eluate to Ethanol (v/v)	% of Recovered Activity			Specific Activity (units/mg)		
	pH 5.8	pH 7.0	pH 8.0	pH 5.8	pH 7.0	pH 8.0
ppt 1:1	40	40	29	11,960	14,400	9170
ppt 1:2	55	59	69	19,500	26,250	19,810
ppt 1:4	5	1	2	37,780	5080	8570



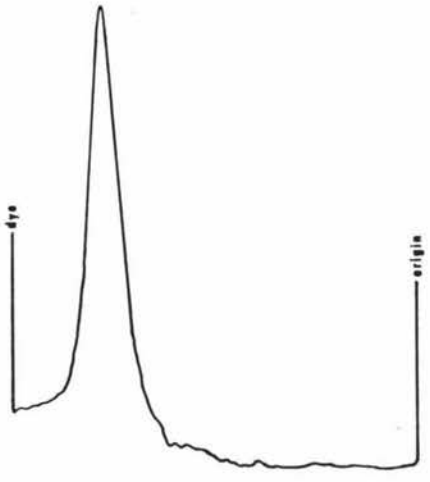
pH 5.8, 10 µg



pH 8.0, 13 µg



pH 7.0, 10 µg



pH 7.0, 5 µg

Fig. 15 SDS gel electrophoresis of 1:2 pancreas precipitates following ethanol precipitation at pH 5.8, 7.0 and 8.0

4.3.2.2 Criteria of Purity of Pancreas Preparations

SDS gel electrophoresis was performed on various pancreas preparations throughout the study to monitor their purity. For example, Fig. 16 shows the gel scan of a 15 μg sample of pancreas, precipitate 1:2 (19,130 units/mg) (1), which was used to immunize rabbits C and D. Whereas this preparation contained only one component, a 3 μg sample of pancreas, precipitate 1:2 (11,190 units/mg) (2), which was also used to immunize rabbits C and D as described in Methods 3.13.1, showed two components (Fig. 16).

4.4 Standard Preparation of the Antibacterial Factor

4.4.1 Bovine Seminal Plasma

4.4.1.1 Sample Calculation

A sample calculation of the recovery of the antibacterial factor from bovine seminal plasma during the isolation procedure follows:

	Activity ($units/cm^3$)	Total Activity (units)	Protein (mg/cm^3)	Total Protein (mg)	Specific Activity (units/mg)
1) BSP (100 cm^3)	21,250	2,125,000	58.6	5860	360
2) CP treatment of BSP yielded:					
first supernatant	2,200	330,000			
buffer wash 1	530	51,940			
NaCl wash 1	640	140,800			
NaCl wash 2	not detected	-			
buffer wash 2	not detected	-			
final supernatant	12,000	3,516,000	3.0	879	4000
total units recovered from CP treatment (supernatants + washes)		4,038,740			

% of activity recovered from CP treatment in final supernatant = 87%
% recovered from BSP in final supernatant = 190% of the BSP activity
= 15% of the BSP protein.

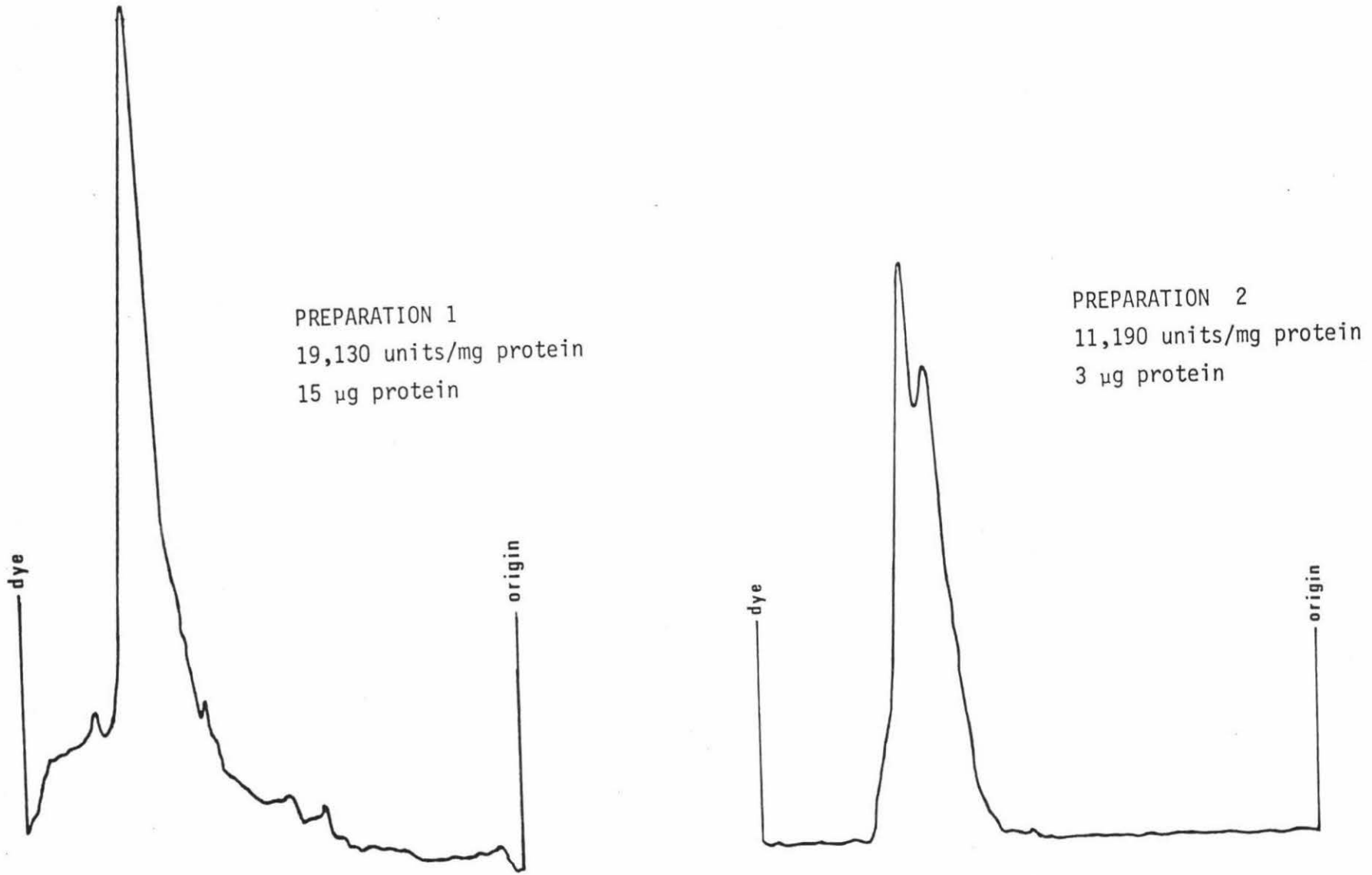


Fig. 16 SDS gel electrophoresis of pancreas preparations used to immunize rabbits C and D.

3) ethanol precipitation of final CP supernatant yielded:

	total protein (mg)	total activity (units)	specific activity (units/mg)
ppt 1:2	561	1,721,410	3140
ppt 1:4	112	951,140	8470

% recovered from final CP supernatant after precipitation:

	protein	activity
ppt 1:2	64	49
ppt 1:4	13	27
Sum	77	76

4) minimum calculated number of units/cm^3 based on the number of units recovered from CP treatment = 4,038,740 units/100 cm^3 BSP

= 40,387 units/cm^3 BSP

assuming one protein is responsible for the antibacterial activity, and as the S.A. of ppt 1:4 = 8472 units/mg then:

40,387 units/cm^3 ÷ 8472 units/mg = 4.8 mg factor/cm^3 BSP

= 8.2% of total BSP protein

= 480 mg factor/100 cm^3 BSP

ppt 1:4 contained 112 mg protein = 23% of the factor calculated to be present in BSP.

5) ethanol reprecipitation of the aqueous 1:4 solution yielded:

	total protein (mg)	total activity (units)	specific activity (units/mg)
ppt 1:2[1]	66.7	758,620	11,380
1:3[1]	5.7	105,750	18,400
1:5[1]	0.5	6,780	26,800
Sum	72.9	871,150	

92% of the activity and 59% of the protein was recovered from the reprecipitation of precipitate 1:4.

6) The calculated yield of factor depends on the specific activity of the preparation. Thus, the preceeding calculations (4) were repeated using an average specific activity, determined from the total units/total mg protein recovered in the 1:2[1], 1:3[1], and 1:5[1] precipitates.

average specific activity = 871,150 units/72.9 mg
= 11,950 units/mg

40,387 units/cm^3 ÷ 11,950 units/mg = 3.4 mg factor/cm^3 BSP
= 5.8% of total BSP protein
= 340 mg factor/100 cm^3 BSP

the 3 precipitates contained 72.9 mg = 21% of the factor calculated to be present in BSP
= 1.2% of total BSP protein.

The purification achieved was:

	sample	specific activity (units/mg)	purification
1.	BSP	360	1
2.	final CP eluate	4000	11 x
3.	ppt 1:4	8470	23 x
4.	ppt 1:2[1]	11,380	31 x
5.	ppt 1:3[1]	18,400	51 x

4.4.1.2 Summary of Standard Preparations : Yield and Purification

Six, large batch cellulose phosphate treatments of BSP yielded an average recovery of 166% of the initial BSP activity. That is, an increase in activity of 66% was obtained. The results obtained from five standard preparations of BSP antibacterial factor are presented in Table XXV. The calculated amount of factor present in BSP was based on the specific activities of the 1:4 precipitates, and in the third preparation, on the average specific activity of precipitates 1:3 and 1:7 as determined by total units/total mg protein.

Table XXV. Summary of recovery of antibacterial factor from BSP

Recovery of Antibacterial Factor		Preparation I	II	III	IV	V	Average
calculated activity (units/cm) in BSP		48,660	45,630	69,270	40,390	33,710	47,530
% of total recovered activity in final CP eluate		58	89	89	87	93	83
% of BSP protein in final CP eluate		18	21	6	15	14	15
% of BSP activity in final CP eluate		97	133	147	165	118	132
% of final CP eluate protein in ppt:	1:2	77	49	120	64	99	82
	1:3	-	-	3	-	-	
	1:4	9	7	-	13	13	10
	1:7	-	-	6	-	-	
% of final CP eluate activity in ppt:	1:2	35	81	55	49	41	52
	1:3	-	-	4	-	-	
	1:4	40	36	-	27	25	29
	1:7	-	-	11	-	-	
calculated % of protein in BSP which is factor		7.3	4.6	4.0	8.2	8.0	6.4
ave. specific activity (units/mg) of best fractions		9960	8430	10,980	8470	8980	9360
% of calculated total activity recovered in best fraction(s)		23	32	13	23	23	23
% of total BSP protein in best fraction(s)		1.7	1.5	0.5	1.9	1.8	1.5
mg factor/100 cm^3 BSP		122	172	82	112	86	96

The yield, of course, depends on the specific activity chosen. Thus, the average yield of antibacterial factor, which had an average specific activity of 9400 units/mg, was determined from five preparations to be 96 mg/100 cm^3 of bovine seminal plasma. However, the average yield of factor having an average specific activity of 16,500 units/mg was 21 mg/100 cm^3 of BSP.

The data illustrate the large amount of activity (52%) which was present in precipitate 1:2. Later experiments presented in Results 5.15.1.1 attempted to recover some of this active material in a more purified form.

The purification achieved for each preparation is presented in Table XXVI. The average specific activity of the cellulose phosphate supernatant was 11 times greater than that of whole BSP. Precipitates in the range 1:3 to 1:7 had specific activities about 32 times greater than the specific activity of BSP. Higher specific activities were achieved following the reprecipitation of precipitate 1:4. In one case, a specific activity 74 times greater than that of BSP was achieved.

An average purification scheme for BSP antibacterial factor may be presented as follows:

	Sample	Specific Activity (units/mg)	Purification
1.	BSP	300	1
2.	final CP eluate	3000	10 x
3.	ethanol precipitate	9000	30 x
4.	ethanol precipitate[1]	17,000	60 x

Table XXVI. BSP preparations : specific activities and purification

Sample	Specific Activity (units/mg) (Purification of BSP)				
	I	II	III	IV	V
BSP	390 (1)	260 (1)	260 (1)	360 (1)	610 (1)
final CP eluate	2090 (5x)	1610 (6x)	6440 (25x)	4000 (11x)	4810 (8x)
ppt 1:2	950 (2x)	2730 (10x)	2990 (12x)	3140 (9x)	2000 (3x)
ppt 1:3	-	-	9500 (37x)	-	
ppt 1:4	9960 (26x)	8430 (32x)	-	8470 (24x)	
ppt 1:7	-		11,620 (45x)	-	
ppt 1:2[1]	4720 (12x)			11,380 (32x)	8740 (14x)
ppt 1:3[1]	-			18,400 (51x)	-
ppt 1:4[1]	17,370 (45x)			-	10,560 (17x)
ppt 1:5[1]	-			26,820 (74x)	-
ppt 1:7[1]	13,930 (36x)			-	-

4.4.2 Pancreas

4.4.2.1 Summary of Standard Preparations : Yield and Purification

Similar calculations (Results 4.4.1.1) were done to determine the recovery of the antibacterial factor from pancreas. The results obtained from four standard preparations are presented in Table XXVII.

The calculated amount of factor present in the pancreas extract (Table XXVII) was based on the specific activities of precipitates 1:2 for preparations I and II, an average specific activity of precipitates 1:1.5 and 1:2.5 for preparation III, and an average specific activity of precipitates 1:1.5 and 1:2 for preparation IV. The average specific activities were determined as total units/total mg protein of the precipitates used in the calculation. The yield of factor was based on the total protein of the fractions which were used to determine the calculated amount of factor present.

The average yield from four pancreas preparations of antibacterial factor, which had an average specific activity of 20,000 units/mg, was 38 mg/100 g tissue (Table XXVII).

As discussed previously (Results 4.3.2), the data from the standard preparations (Table XXVII) indicate, that the greatest proportion of the activity, precipitates in and before the 1:1.5 ethanol cut, as shown in preparations III and IV.

The purifications achieved for each preparation are presented in Table XXVIII. The average specific activity of the cellulose phosphate final eluate was about 8 times greater than that of the pancreas extract. The ethanol precipitation did not increase the specific activity greatly, and the best fractions had specific activities about 12 times greater than that of the crude extract, although in one case a 21 fold increase in

Table XXVII. Summary of recovery of antibacterial factor from pancreas extracts

Recovery of Antibacterial Factor		Preparation I	II	III	IV	Average
activity (units/cm^3) in pancreas extract		22,250	24,500	22,250	24,500	23,380
% of total recovered activity in final CP eluate		53	30	53	58	48
% of extract protein in final CP eluate		5	6	5	6	6
% of extract activity in final CP eluate		63	23	51	35	43
% of final CP eluate protein in ppt:	1:0.75	-	-	-	46	
	1:1	42	36	-	-	
	1:1.5	-	-	67	22	
	1:2	33	25	-	5	
	1:2.5	-	-	10	-	
	1:4	-	-	2	7	
% of final CP eluate activity in ppt:	1:0.75	-	-	-	40	
	1:1	37	18	-	-	
	1:1.5	-	-	58	29	
	1:2	54	54	-	6	
	1:2.5	-	-	10	-	
	1:4	-	-	2	6	
calculated % of protein in pancreas extract which is factor		5.2	12.2	9.3	12.8	9.9
ave. S.A (units/mg) of best fractions		26,250	19,130	14,580	18,280	19,560
% of calculated total activity recovered in best fraction(s)		34	13	43	12	25
% of total extract protein in best fraction(s)		1.8	1.5	4.0	1.6	2.2
mg factor/100 g pancreas tissue		34	20	79	21	38

Table XXVIII. Pancreas preparations : specific activities and purification

	Specific Activity (units/mg) (Purification of Extract)			
Sample	I	II	III	IV
pancreas extract	1220 (1)	2330 (1)	1220 (1)	2330 (1)
final CP eluate	13,000 (11x)	9050 (4x)	16,500 (14x)	14,350 (6x)
ppt 1:0.75	-	-	-	12,590 (5x)
ppt 1:1	14,400 (12x)	4670 (2x)	-	-
ppt 1:1.5	-	-	14,350 (12x)	18,410 (8x)
ppt 1:2	26,250 (22x)	19,130 (8x)	-	16,670 (7x)
ppt 1:2.5	-	-	16,000 (13x)	-
ppt 1:4	-	-	6230 (5x)	12,140 (5x)

specific activity was recorded.

An average purification scheme for pancreas antibacterial factor may be presented as follows:

	Sample	Specific activity (units/mg)	Purification
1.	crude pancreas extract	1700	1
2.	final CP eluate	13,000	8 x
3.	ethanol precipitate	20,000	11 x

4.5 Ammonium Sulphate Fractionation of BSP and Pancreas-Cellulose Phosphate Eluates

Ammonium sulphate is commonly used for protein precipitation, and therefore, fractionations of final CP eluates from BSP and pancreas extract were attempted (Methods 2.5). Both eluates were diluted with 0.01M sodium phosphate buffer, pH 7.4. The BSP eluate had 4.2 mg protein/cm^3 and 4400 units/mg, and the pancreas eluate had 0.3 mg protein/cm^3 and 4050 units/mg. The activity of each fraction except the final supernatant, which contained 2.6M $(NH_4)_2SO_4$, was determined before dialysis.

Dialysis was necessary to reduce the ammonium sulphate to acceptable levels for protein determination by the method of Lowry et al. (1951). Although ammonium sulphate did not exhibit antibacterial activity up to 2.0M, 3.0M $(NH_4)_2SO_4$ caused a diffuse zone of inhibition against M. lysodeikticus in the agar diffusion assay.

The protein and activity determinations of each fraction following dialysis are presented in Table XXIX. Most of the active material from BSP precipitated between 40 and 70% $(NH_4)_2SO_4$ saturation. The active material from pancreas was, on the other hand, largely soluble, even at 70% $(NH_4)_2SO_4$ saturation.

Approximately 50% of the activity was lost following dialysis. Of

Table XXIX. Ammonium sulphate fractionation of BSP and pancreas eluates

% $(NH_4)_2SO_4$ Saturation	% of Initial Activity		% of Initial Protein		% of Total Activity Recovered		% of Total Protein Recovered		Specific Activity (units/mg)		% Activity Lost After Dialysis	
	BSP	Panc	BSP	Panc	BSP	Panc	BSP	Panc	BSP	Panc	BSP	Panc
0 - 20	ND*	ND*	0.3	0.1	-	-	0.6	0.2	-	-	-	-
20 - 30	ND*	ND*	0.1	0.5	-	-	0.3	0.6	-	-	-	-
30 - 40	0.3	ND*	0.8	1.1	0.7	-	1.7	1.8	1750	-	67	-
40 - 50	11.1	ND*	6.7	-	23.5	-	14.1	-	7320	-	60	-
50 - 60	14.3	3.6	6.7	8.0	30.5	10.9	14.1	13.2	9430	6000	44	51
60 - 70	17.3	4.5	6.9	7.5	37.0	13.9	14.5	12.4	11,110	8210	49	46
final sup^n	3.8	24.5	26.0	43.3	8.2	75.1	54.8	71.6	650	7640		
Sum	46.8	32.6	47.5	60.5								

*ND = no activity detected.

the remaining 50%, 47% (BSP) and 33% (pancreas) were recovered. Some of the fractions may have contained activity, which was not detected at the concentrations assayed.

The effect of ethanol precipitation, following the ammonium sulphate fractionation of a final BSP-CP eluate, was determined. A 40 to 70% $(NH_4)_2SO_4$ fraction of the eluate (4.2 mg protein/cm^3, 4400 units/mg) was obtained and resuspended in 0.01M sodium phosphate buffer, pH 7.4. An ethanol precipitation of this fraction is presented in Table XXX. Seventy-six per cent of the activity used in the precipitation was recovered.

Table XXX. Ethanol precipitation of a 40 to 70% ammonium sulphate fraction of a BSP-CP eluate.

Ratio of $(NH_4)_2SO_4$ Fraction To Ethanol (v/v)		% Recovered Activity	Specific Activity (units/mg)
ppt	1:1	4	2470
ppt	1:3	82	8370
ppt	1:4	10	10,440
ppt	1:7	4	9710

With respect to the specific activities of the various ethanol fractions, following ammonium sulphate fractionation, no increase was observed over those fractions obtained directly by ethanol precipitation of the final CP eluate. Consequently, an ammonium sulphate fractionation was not used in the purification procedure.

5. Characterization of Antibacterial Factors from Bovine Seminal Plasma and Pancreas

5.1 Dialysis of Ethanol Precipitated Material

5.1.1 Bovine Seminal Plasma Preparation

Four aliquots of a BSP 1:4 precipitate (1.5 mg protein/cm^3, 26,500 units/cm^3) were dialyzed at 4^o against 200 volumes of distilled water. At various intervals over 24 h, a sample was removed and analyzed, and the water was replaced. Fig. 17 illustrates the per cent protein and activity remaining, following dialysis. The loss of protein was paralleled by the loss of activity, which decreased to 13% of the original following dialysis for 24 h. The dialyzability of the active protein suggested that it had a relatively low molecular weight.

5.1.2 Pancreas Preparation

Purified pancreas 1:2 precipitates contained high concentrations of citrate. In order to reduce the citrate concentration, two aliquots of a precipitate solution, containing 50.4 mg citrate/cm^3, were dialyzed against 150 volumes of 0.01M sodium phosphate buffer, pH 7.4. One sample was removed after 5 h and the second after 22 h. The results (Table XXXI) show that although 94% of the citrate was lost after 5 h, 63% of the activity was also lost. In a second experiment, in which samples were dialyzed for shorter intervals but against 0.05M sodium phosphate buffer, pH 7.4, similar results were obtained (Table XXXI).

Table XXXI. Loss of citrate, protein and activity following dialysis of pancreas, 1:2 ethanol precipitates against buffer

% Loss of	I. Dialysis Against 0.01M Sodium Phosphate Buffer, pH 7.4		II. Dialysis Against 0.05M Sodium Phosphate Buffer, pH 7.4		
	5 hours	22 hours	2 hours	4 hours	6 hours
Citrate	94	99	70	88	95
Protein	13	13	12	12	12
Activity	63	76	59	69	69

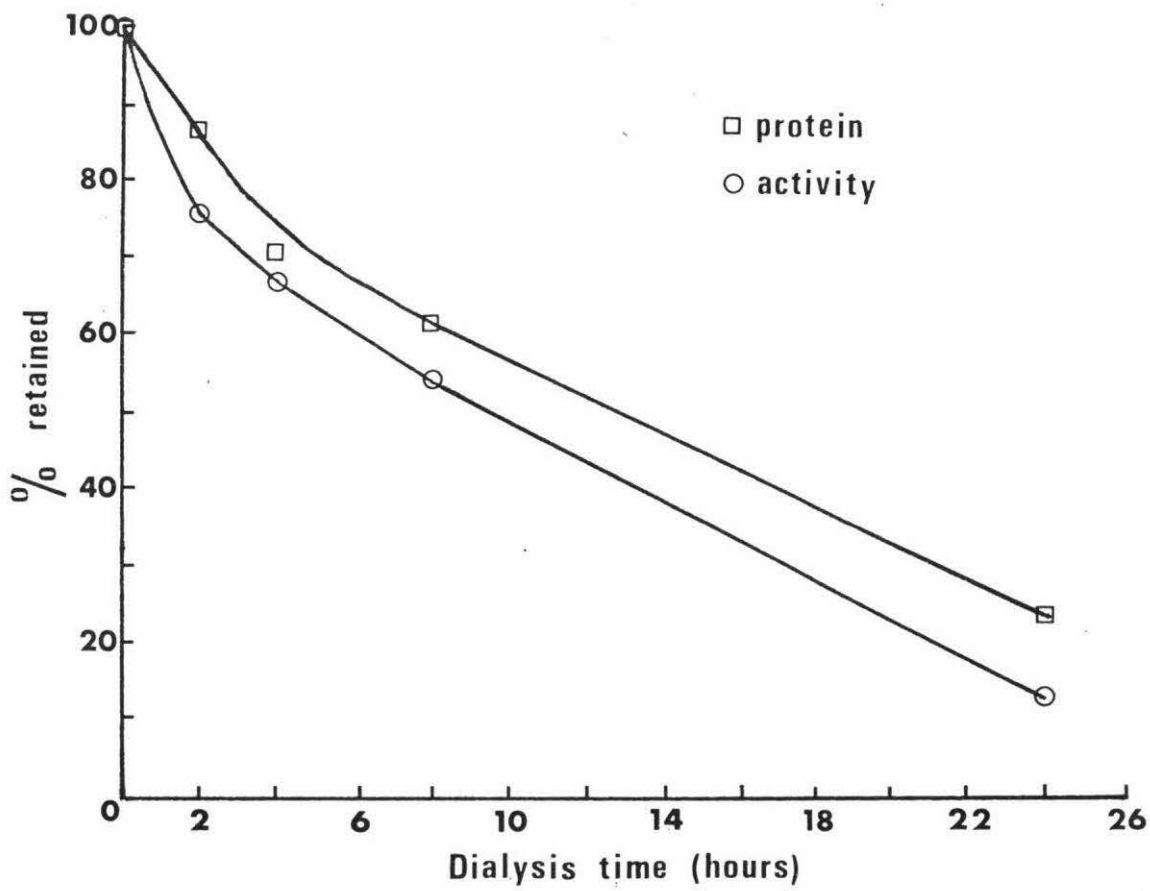


Fig. 17 Loss of protein and activity following dialysis of a solution of a BSP, 1:4 ethanol precipitate against distilled water

The dialysis of a large volume (70 cm^3) of a 1:2 precipitate solution, against 30 volumes of 0.01M phosphate buffer, pH 7.4, for 4 h, resulted in a loss of 41% of the protein and 51% of the activity. In contrast, dialysis of smaller volumes (4 cm^3) resulted in a loss of only 13% of the protein but 69% of the activity. This discrepancy could reflect the variability of the dialysis tubing, since a tubing with a greater diameter was used for the dialysis of the larger volume. The dialyzability of the pancreas active material, like the purified BSP material, suggested a substance(s) with a low molecular weight.

5.2 Determination of Molecular Weight

5.2.1 BSP Preparation

Three molecular weight determinations were performed on purified BSP factor using SDS gel electrophoresis (Methods 3.1.5). An example of a plot of known molecular weight markers versus relative mobilities is presented in Fig. 18. The average molecular weight determined for the purified BSP antibacterial factor was 15,250 ($\pm$ 10%) daltons.

5.2.2 Pancreas Preparation

For comparison, the molecular weight was determined for the isolated pancreas material. The main component of the preparation analyzed had a molecular weight of 15,000 ($\pm$ 10%) daltons, and the lesser component had a molecular weight of 16,250 ($\pm$ 10%) daltons.

Electrophoresis, conducted for one-third and one-half the time of a normal run, insured that no very small molecular weight components were present in either the BSP or pancreas preparations, as no protein band was detected ahead of the dye front.

5.3 Ultraviolet Absorption

The ultraviolet absorption pattern for both the purified BSP and

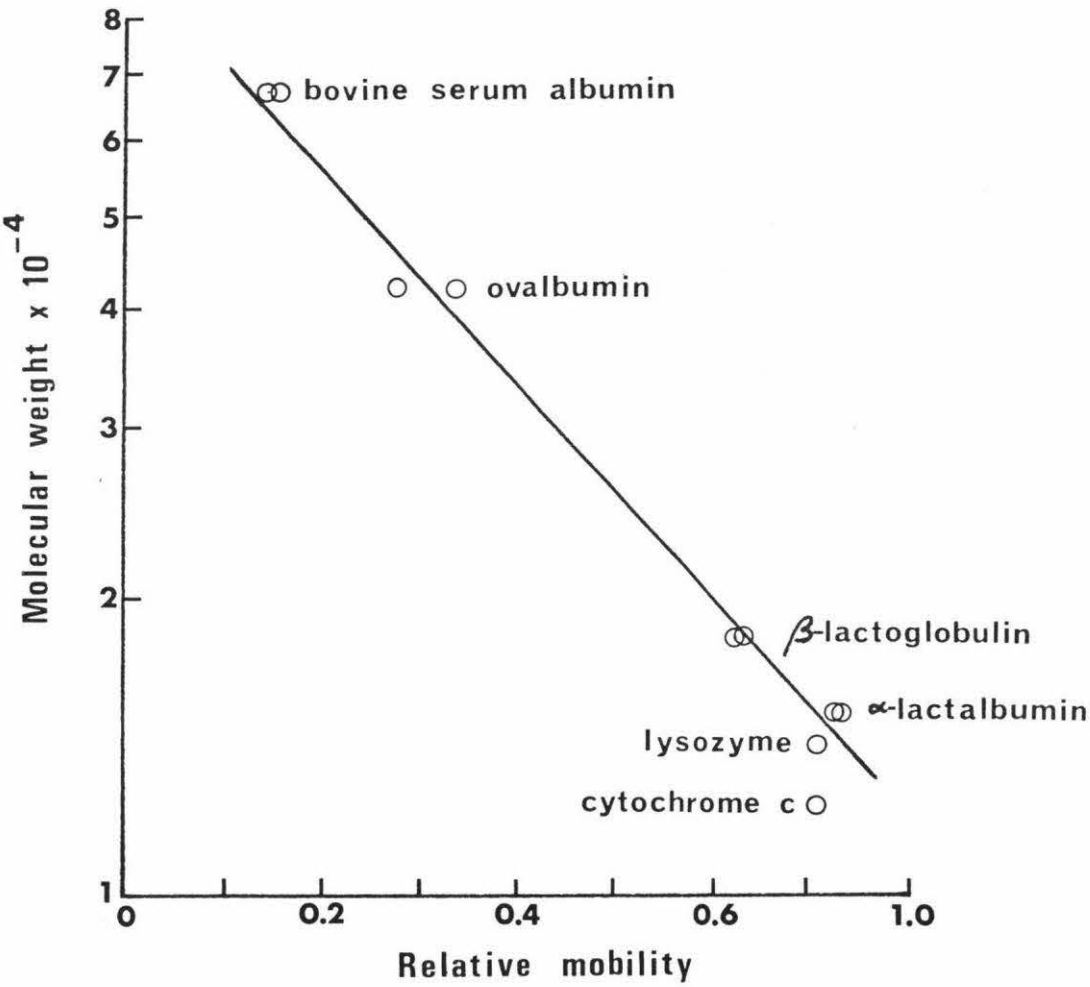


Fig. 18 Determination of molecular weight by SDS gel electrophoresis

pancreas preparations analyzed, showed a small broad peak at 280 nm, and a much larger peak at 225 nm. Both peaks are indicative of the aromatic acids, tyrosine, phenylalanine and tryptophan (Wetlaufer, 1962), and the latter peak also represents absorption by the peptide bonds of proteins in general (Wetlaufer, 1962). The absorption scans demonstrated that neither the BSP nor the pancreas factor is a nucleic acid-protein complex.

5.4 Amino Acid Analysis of a BSP Preparation

Table XXXII presents the average amino acid composition of a BSP preparation (18,400 units/mg) from duplicate analyses. The values are expressed as moles per 100 moles of amino acids recovered. The lysine/arginine ratio for the preparation was 1.27.

The minimum molecular weight was calculated as follows:

Sum (no. of residues x molecular weight of amino acid) - 18 (no. of residues - 1).

The correction was made to compensate for water added on during the hydrolysis of the peptide chain to liberate free amino acids.

If methionine is set as one residue per peptide molecule, then the minimum molecular weight of the material is calculated as 26,400 daltons. However, if the preparation also contained small amounts of contaminating proteins, then the methionine value might be higher than the true value for the factor.

If cystine/2, which is the amino acid with the next lowest number of residues, is set as one residue per peptide molecule, then the molecular weight is 16000 daltons. Considering the error in this value due to the presence of small amounts of contaminating proteins, this value approximates that obtained by SDS gel electrophoresis (15,250 $\pm$ 10% daltons).

Table XXXII. Amino acid analysis of a BSP preparation

amino acid	molar %	molar % assuming one methionine residue per peptide molecule	molar % assuming one cystine/2 residue per peptide molecule
lysine	11.4	25.0	16.3
histidine	4.2	9.2	6.0
arginine	9.0	19.8	12.9
asp, asp^{n}	12.2	26.8	17.4
threonine	2.7	6.0	3.9
serine	10.9	23.9	15.6
glu, glu^{n}	4.9	10.8	7.0
proline	4.8	10.6	6.9
glycine	7.2	15.8	10.3
alanine	8.0	17.6	11.4
cystine/2	0.7	1.6	1.0
valine	2.2	4.8	3.1
methionine	0.45	1.0	0.6
isoleucine	2.5	5.4	3.6
leucine	12.4	27.4	17.7
tyrosine	2.6	5.8	3.7
phenylalanine	4.1	9.0	5.9
no. of residues		220.5	142.7
molecular weight		26,400	16,000

5.5 Comparison of BSP Preparation with Histones

A comparison of the BSP preparation and the four major classes of histones (Elgin and Weintraub, 1975) is presented in Table XXXIII. With regard to the lysine/arginine ratio and molecular weight, the BSP material appears similar to the histone group of proteins, especially fractions H2a, H2b, H3 and H4.

Table XXXIII. Comparison of the BSP fraction and the calf thymus histones, as reviewed by Elgin and Weintraub (1975).

Class	Fraction	Lysine/arginine	Molecular Weight
very lysine rich	H1 (f1)	22.0	~21,500
lysine rich	H2a (f2a2)	1.17	14,004
	H2b (f2b)	2.50	13,774
arginine rich	H3 (f3)	0.72	15,324
	H4 (f2a1)	0.79	11,282
BSP preparation		1.27	~15,000

A comparison of the amino acid composition of the BSP preparation and the various histones from calf thymus is presented in Table XXXIV. The amino acid composition of the BSP preparation differs from any of the histone fractions presented. For example, a comparison of the BSP fraction and histone H2a shows that although many amino acids occur in similar amounts, in most cases, aspartic acid, serine and phenylalanine are much higher, and valine and alanine are lower in the BSP material. No cystine/2 was found in the whole histone or in any of the histone fractions shown, except H3, whereas it was present in the BSP fraction.

Table XXXIV. Comparison of amino acid analyses of the BSP fraction and calf thymus histones

Amino Acid	Moles/100 Moles of Amino Acids Recovered						
	BSP fraction	H1	H2a	H2b	H3	H4	Whole histone
lysine	11.4	25.6	10.8	16.0	9.6	10.7	15.6
histidine	4.2	0.1	3.1	2.4	1.5	2.0	2.0
arginine	9.0	2.5	9.3	6.4	13.3	13.7	9.2
asp, asp^n	12.1	3.1	6.2	4.8	3.7	4.9	14.2
glu, glu^n	4.9	5.3	9.3	8.0	11.1	5.9	
threonine	2.7	5.6	3.9	6.4	7.4	6.7	5.8
serine	10.9	6.4	3.1	11.2	3.7	2.0	5.2
proline	4.8	9.7	3.9	4.8	4.4	1.0	5.1
glycine	7.2	7.4	10.8	5.6	5.2	16.7	8.5
alanine	8.0	23.3	13.2	10.4	13.3	6.9	13.8
cystine/2	0.7	-	-	-	1.5	-	-
valine	2.2	4.5	6.2	7.2	4.4	8.8	6.4
methionine	0.4	-	-	1.6	1.5	1.0	-
isoleucine	2.4	0.9	4.6	4.8	5.2	5.9	10.6
leucine	12.5	4.0	12.4	4.8	8.9	7.8	
tyrosine	2.6	0.6	2.3	4.0	2.2	3.9	1.7
phenylalanine	4.1	0.7	0.8	1.6	3.0	2.0	1.9
lysine/arginine	1.27	10.24	1.17	2.50	0.72	0.79	1.70
basic/acidic	1.5	3.4	1.5	1.9	1.6	2.4	1.9
reference*		1	2	2	2	2	3

* 1 Kincade and Cole (1966).
2 Elgin and Weintraub (1975).
3 Johns et al. (1960).

5.6 Antibacterial Activity by Broth Dilution (MIC)

5.6.1 BSP Preparations

Table XXXV summarizes the minimal inhibitory concentrations of various BSP preparations in nutrient broth. Within the limitations of doubling dilutions, there was an inverse relation between the specific activity and the MIC of a preparation; that is, the lower the specific activity of a preparation, the higher its MIC. The average minimal inhibitory concentration for the purified BSP preparations was 18 µg protein/cm^3. The average minimal inhibitory concentration for polylysine (Sigma), a synthetic, basic polypeptide, was 8 µg/cm^3 nutrient broth.

5.6.2 Pancreas Preparation

The MIC obtained for purified pancreas material with a specific activity of 11,190 units/mg was 7 µg protein/cm^3. As only one sample was assayed, no correlation between specific activity and minimal inhibitory concentration could be made.

5.6.3 Effect of Media Composition on the MIC of the BSP Factor

The minimal inhibitory concentration of a BSP preparation with a specific activity of 11,380 units/mg was determined in duplicate in nutrient broth, nutrient broth plus 0.3% yeast extract, antibiotic medium 3, trypticase soy broth and brain heart infusion broth (Materials 3.1). The average MIC concentrations obtained were 15, 8, 38, 72 and 72 µg/cm^3 respectively. Thus, the antibacterial activity was decreased in the more complex media; this illustrates the necessity for well-defined conditions in the determination of the MIC of a given preparation.

5.6.4 Effect of pH on the MIC of the BSP Factor

Skarnes and Watson (1956a) used phosphate buffered broth at various

Table XXXV. Minimal inhibitory concentration of BSP preparations in nutrient broth

Sample	Specific Activity (units/mg)	MIC ($\mu g/cm^3$)		Specific Activity x MIC x 10^{-3} (units/cm^3)
ppt 1:2[1]	8740	28		245
ppt 1:2[1]	11,380	9		
	"	10		
	"	15	13.5	154
	"	20		
ppt 1:4[1]	10,560	22		
ppt 1:3	13,320	13	13	173
	"	13		
ppt 1:4	13,850	16		222
average		18		198

pH values, to determine the pH at which the antibacterial peptide from calf thymus exhibited the greatest activity. The MIC of a BSP preparation, with a specific activity of 10,560 units/mg, was determined in duplicate in nutrient broth, nutrient broth in 0.05M sodium phosphate buffer, pH 7.4 and 8.0, and nutrient broth in 0.05M Tris-HCl buffer, pH 7.4 and 8.0. The MIC (μg protein/cm^3) in nutrient broth was 22, in nutrient broth-phosphate buffer, 44, and in nutrient broth-Tris buffer, 88. No difference in the MIC was observed at pH 7.4 and 8.0, in both phosphate and Tris buffers. The increase in MIC observed under buffered conditions might have been due (i) to the enhancement of microbial growth in a buffered system or (ii) to the inhibition of the antibacterial substance by the buffer. Due to the difficulty in attaining adequate controls, the system did not lend itself to the determination of the pH optimum for the antibacterial factor.

5.7 Determination of Lysozyme Activity (Methods 3.5)

The enzymic activity of lysozyme was determined by the hydrolysis of the chromophoric substrate, 3, 4-dinitrophenyl-tetra-N-acetyl-β-chitotetraoside, with the liberation of 3, 4-dinitrophenol which was assayed colorimetrically. The hydrolysis of the substrate by hen egg-white lysozyme is shown in Fig. 19. Two purified BSP preparations (8970 and 10,560 units/mg) and three pancreas preparations (14,400, 26,250 and 11,190 units/mg) at concentrations of 0.05 or 0.1 mg/cm^3, caused no increase in absorbance at 400 nm over 30 min the purified BSP and pancreas materials had no detectable lysozyme activity.

5.8 Determination of Haemolytic Activity

5.8.1 Plate Assay

Crude BSP produced zones of haemolysis on horse, human, sheep and

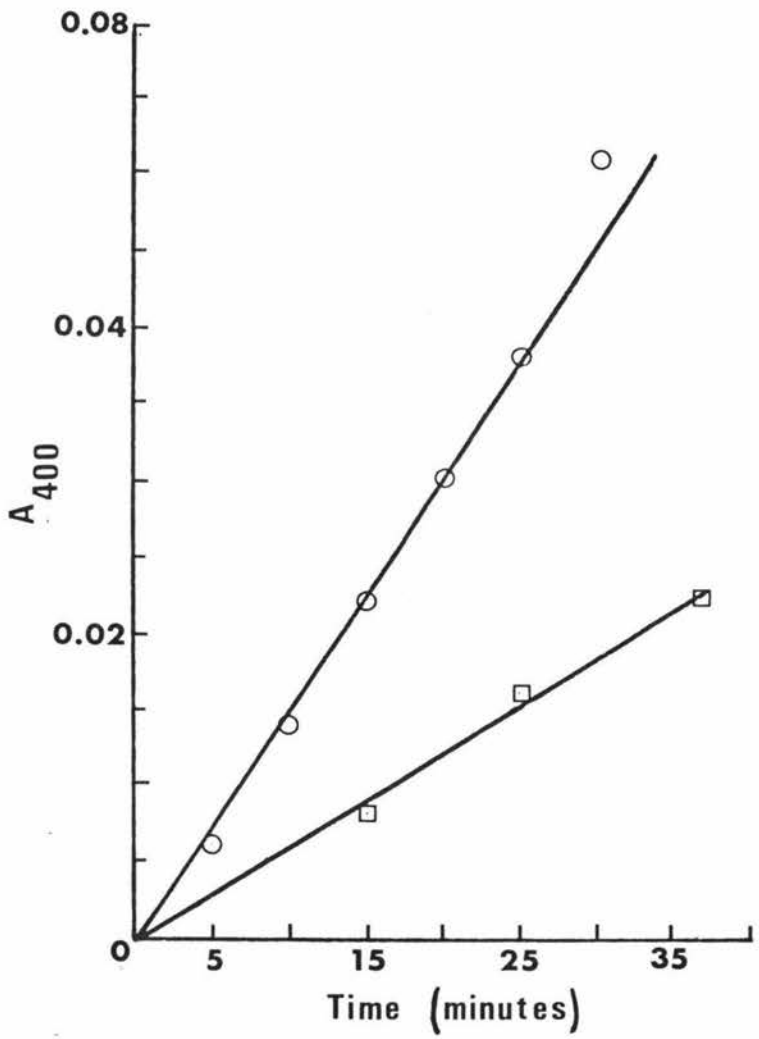


Fig. 19 Hydrolysis of 3,4-dinitrophenyl-glycoside by egg-white lysozyme.

○ 0.1mg lysozyme/cm^3, 0.1mg substrate/0.1 cm^3

□ 0.05mg lysozyme/cm^3, 0.05mg substrate/0.1 cm^3

bovine blood agar (Methods 3.6.1). The largest zone of haemolysis was produced on bovine blood. On chicken blood agar, BSP produced a zone of incomplete haemolysis equal in diameter to the zone produced on bovine blood. A purified BSP preparation (8430 units/mg), however, failed to haemolyse the red blood cells in any of the blood samples.

5.8.2 Tube Assay

Bovine seminal plasma contained 383 haemolytic units/cm^3 as assayed by the method described in Methods 3.6.2. Since the crude BSP had 42,000 antibacterial units/cm^3, then 1 haemolytic unit corresponded to 110 antibacterial units. A purified preparation containing 70,000 antibacterial units/cm^3 should, therefore, contain 636 haemolytic units/cm^3 if the activity of the purified factor corresponds to the haemolytic activity. However, the purified preparation contained only 0.5 haemolytic units/cm^3. These results demonstrate, as did the plate assay results, that the haemolytic activity of BSP did not correspond with the antibacterial activity of a purified preparation.

5.9 Determination of Phospholipase Activity (Methods 3.7)

Crude bovine seminal plasma (116 mg protein/cm^3) exhibited a central, clear zone (phospholipase A-like) surrounded by a somewhat vague, turbid zone (phospholipase C-like) on egg yolk agar, at both 37^o and 50^o. As the purification of BSP proceeded, the clear zone disappeared. The final CP eluate (7.9 mg/cm^3), for example, caused only a vague zone of turbidity. Finally, although one purified BSP preparation (1.7 mg/cm^3) produced a slight turbidity, a second preparation (7.8 mg/cm^3) caused no change on egg yolk agar.

Similarly, whereas crude pancreas extract (16.6 mg/cm^3) caused a small zone of turbidity, a more purified sample, a final CP eluate, showed no activity at the protein concentration tested (0.3 mg/cm^3).

These preliminary results suggested that the phospholipase-like activities of bovine seminal plasma and pancreas extract were not enhanced by the purification process.

5.10 Protein Determinations

A comparison between the Lowry method (Lowry et al., 1951) and the protein-dye binding method (Bradford, 1976), for the determination of the protein concentration of purified preparations of BSP and pancreas extract, showed considerable variation between the two methods. Protein concentrations determined by the dye binding method were 40 to 65% lower than those obtained by the Lowry method.

5.11 Effect of Repeated Freezing and Thawing on the Antibacterial Activity of BSP

Table XXXVI illustrates that the antibacterial activity of bovine seminal plasma remains relatively constant following repeated freezing and thawing; slight variations are only a reflection of the assay system employed.

Table XXXVI. Effect of freezing and thawing on the antibacterial activity of bovine seminal plasma

No. of Times Thawed	Activity (units/cm^3)
1	19,000
2	18,500
3	22,250
4	20,500
5	21,250
6	21,000

5.12 Trypsin Sensitivity

Preliminary experiments (Methods 3.8) demonstrated that, in a reaction mixture containing 2.4 mg purified BSP protein/cm^3 (11,380 units/mg) and 20 µg trypsin/cm^3, 100% of the antibacterial activity was destroyed during incubation at 30^o for 30 min. When the trypsin concentration was reduced by a factor of ten, 52% of the activity was lost.

The boiled controls showed, in most cases, no loss of activity; this eliminated the possibility of inactivation by heat-stable substances which might be present in the trypsin preparation (Hirsch and Dubos, 1954). A slight inactivation which was observed in some of the boiled controls was due to the action of trypsin on the peptide, before complete inactivation of the trypsin had occurred.

Similarly, purified pancreas material was sensitive to trypsin. In a reaction mixture containing 1.1 mg pancreas protein/cm^3 and 20 µg trypsin/cm^3, 100% of the antibacterial activity was destroyed. When the trypsin concentration was reduced by a factor of ten, no loss of activity was observed.

A similar experiment confirmed the sensitivity of both factors to trypsin. Complete inactivation of antibacterial activity was observed in mixtures with final concentrations of 0.9 mg of either preparation/cm^3 and 20 µg of trypsin/cm^3 incubated at 37^o for 30 min.

5.13 Heat Stability

No loss of activity of either purified BSP factor (0.9 mg/cm^3, 10,560 units/mg, pH 7.4) or pancreas factor (1.2 mg/cm^3, 11,190 units/mg, pH 7.6) was observed following 10 min at temperatures in the range 30 to 100^o (Methods 3.9). In contrast, crude bovine seminal plasma, although relatively stable up to 60^o for 10 min, retained only 30% of its activity

following 10 min at 100^{o} as shown in Fig. 20. The loss of antibacterial activity when whole BSP is heated, may be due to binding of the active fraction to denatured proteins. Neither purified BSP nor pancreas factor showed any detectable loss in activity when autoclaved at 120^{o} for 15 min.

5.14 Inhibition of Antibacterial Activity by Deoxyribonucleic Acid

The inhibition of the antibacterial activity of cationic tissue proteins by anionic polymers has been observed by various investigators (Bloom et al., 1951; Burger and Stahmann, 1952; Skarnes and Watson, 1956a; Hirsch, 1958; Zeya and Spitznagel, 1966b; Hibbitt, 1970). For example, acidic macromolecules blocked the antibacterial action of calf thymus tissue peptide when the ratio of macromolecule to peptide was equal or greater (Skarnes and Watson, 1956a). Hirsch (1958) reported that the minimal concentration of DNA, producing reversal of the lethal effect of histone on E. coli K12, was 0.4 µg and 1.0 µg of DNA/cm^3 in mixtures containing 2.0 µg and 4.0 µg of histone/cm^3 respectively.

Hibbitt (1970) recorded complete inhibition of growth of Staphylococcus aureus in the presence of 8.0 µg of teat canal - cationic proteins/cm^3. However, in the presence of 8.0 µg of cationic proteins and 3.2 µg of DNA no inhibition of growth was observed; the nucleic acid blocked the action of the cationic proteins.

For comparison, the effect of DNA on the antibacterial activity of purified BSP material was studied. Due to the anticipated problem of the diffusion in agar of any antibacterial factor bound to DNA, the diffusion assay was not used. Instead, the effect of DNA was determined using the broth dilution technique.

The minimal inhibitory concentration of BSP factor (11,380 units/mg) was doubled from 12.5 to 25.0 µg/cm^3 in the presence of equal proportions

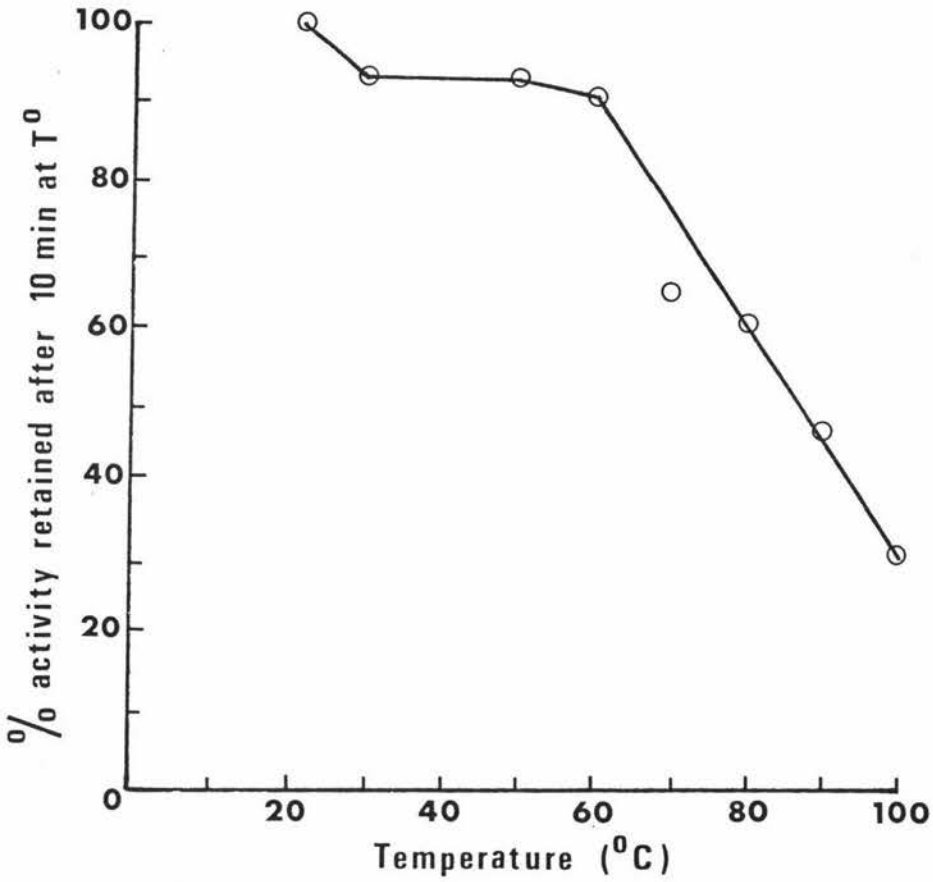


Fig. 20 Effect of temperature on whole bovine seminal plasma

(w/w) of DNA (duplicate determinations) (Methods 3.10). The same results were obtained when the amount of DNA used was 25% that of the peptide. In an additional trial, however, the MIC of the BSP material remained unchanged in the presence of twice the amount of DNA relative to the antibacterial factor. The MIC of purified pancreas material (11,190 units/mg) was increased from 6.2 to 25.0 $\mu g.cm^3$ in the presence of equal proportions of DNA (one determination).

These results indicate that DNA, in approximately equal proportions (w/w) to BSP and pancreas antibacterial proteins, exhibits little or no inhibitory activity towards these antibacterial factors.

5.15 Comparison of BSP Ethanol Precipitates 1:2 and 1:4

5.15.1 Ethanol Reprecipitation Under Various Conditions

5.15.1.1 Direct Reprecipitation of a Solution of 1:2 Ethanol Precipitated Material

Reprecipitation experiments were attempted initially, to recover some of the activity left in the 1:2 precipitate obtained by ethanol precipitation of the final CP eluate. A direct reprecipitation (Methods 3.11.1) of an aqueous solution of a 1:2 precipitate (to yield a $1:2^1$ precipitate) resulted in 39% of the activity remaining in the supernatant. However, the specific activity of the supernatant was comparable to that of the first 1:2 precipitate. It seemed reasonable to propose that contaminating proteins and the active protein had both remained soluble at 67% ethanol. Thus, reprecipitation appeared to be of little value in the recovery of material with specific activity equivalent to that of the initial 1:4 precipitate as shown below:

		specific activity (units/mg)
1.	1st ppt, 1:2	950
2.	1st ppt, 1:4	8970
3.	2nd ppt, 1:2[1]	750
4.	supernatant after reprecipitation	980

In this initial reprecipitation, 100% of the protein and activity were recovered.

5.15.1.2 Reprecipitation of Solutions of 1:2 Ethanol Precipitated Material Following Acidification

The initial reprecipitation experiment suggested that some or all of the antibacterial activity, which precipitated at 67% ethanol (ppt 1:2), may be coprecipitating in a bound form with other proteins. Acidification of the precipitated material could, perhaps, separate the active protein from contaminating proteins. With this in mind, three 1:2 precipitates, which were each resuspended in water to a volume equal to 83% of that of the eluates from which they were originally precipitated (approximately 65 mg citrate/cm^3), were acidified and neutralized as described in Methods 3.11.1. Aliquots from each of the three solutions were then reprecipitated with ethanol, in an attempt to recover activity with a high specific activity (Table XXXVII).

In the first run, an aliquot of each supernatant was saved for analysis. However, the results which are presented, were based on calculations which assumed that the entire supernatant was used for the succeeding precipitation. In the second and third runs, the entire supernatant was precipitated at each stage, and no correction was needed. Also, in these runs, a 1:7 precipitate was not collected.

Table XXXVII. Ethanol reprecipitation of solutions of 1:2 ethanol precipitated BSP material (I, II, III) and 1:4 ethanol precipitated material (IV) following acidification

Recovery of Activity and Protein	I	II	III	average %	IV
total % activity recovered from ppt 1:2	34	20	27	27	51
% of Total Activity In:					
ppt $1:2^{1}_{acid}$	ND*	ND*	ND*	-	36
ppt $1:4^{1}_{acid}$	9	18	12	13	ND*
ppt $1:7^{1}_{acid}$	3	-	-	-	-
final supernatant$_{acid}$	77	82	88	83	64
total % protein recovered from ppt 1:2	91	72	79	81	87
% of Recovered Protein In:					
ppt $1:2^{1}_{acid}$	68	62	54	61	19
ppt $1:4^{1}_{acid}$	16	16	30	21	0.4
ppt $1:7^{1}_{acid}$	2	-	-	-	-
final supernatant$_{acid}$	14	21	16	17	66
Specific Activity (units/mg)					
initial ppt 1:4	8430	8470	11,620		11,620
initial ppt 1:2	2730	3140	2990		-
ppt $1:2^{1}_{acid}$	-	-	-		11,280
ppt $1:4^{1}_{acid}$	7440	920	400		-
ppt $1:7^{1}_{acid}$	2240	-	-		-
final supernatant$_{acid}$	5760	3170	5680		5780

* ND = no activity detected.

In summary, an average of 27% of the initial activity of the 1:2 precipitate was recovered, and of this activity, 83% remained in the final supernatant following a $1:4^{1}_{acid}$ precipitation. However, an average of 81% of the initial protein was recovered, with 61% occurring in the $1:2^{1}_{acid}$ ppt.

In the first run, 77%, 58% and 34% of the initial activity remained, following the $1:2^{1}_{acid}$, $1:4^{1}_{acid}$ and $1:7^{1}_{acid}$ precipitations, respectively. Thus, the loss of activity observed in the reprecipitations following acidification, was not simply a reflection of the acidic treatment, because the amount of recoverable activity decreased as the precipitation process continued.

None of the fractions showed specific activities equivalent to the initial 1:4 precipitates.

5.15.1.3 Acetone Precipitation of the Final Supernatant Obtained after Ethanol Reprecipitation Following Acidification

In the third run, described in Results 5.15.1.2, the addition of acetone to an aliquot of the final supernatant showed the activity to be precipitated by acetone (ppt $1:3^{1}$). A greater per cent of the activity could be accounted for following the acetone precipitation. That is, whereas 23% of the activity was recovered in the final supernatant following analysis of a sample after lyophilization, 42% of the initial activity was recovered in the acetone precipitate from the final supernatant. Calculations were based on the acetone precipitation of the entire supernatant. The acetone precipitate was not lyophilized, which may account for the greater recovery.

5.15.1.4 Reprecipitation of a Solution of 1:4 Ethanol Precipitated Material Following Acidification (Methods 3.11.2)

The citrate concentration of an aqueous solution of a 1:4 precipitate was adjusted to 65 mg/cm^3. The solution was acidified and neutralized, and 10 cm^3 was precipitated with ethanol; the profile obtained (ppt $1{:}2^1_{acid}$, $1{:}4^1_{acid}$) was compared to those of 1:2 precipitates which had been treated similarly (Table XXXVII).

With regard to recovery, 87% of the protein and 51% of the activity was accounted for. Although the protein recovery was comparable to that following the treatment of precipitate 1:2, the antibacterial activity recovered was approximately 30% higher than that recovered from precipitate 1:2.

Differences in the reprecipitation profiles of the 1:2 and 1:4 precipitates were obvious. Whereas the majority of the activity (64%) occurred in the final supernatant$_{acid}$ following reprecipitation of precipitate 1:4, 36% of the recovered activity was present in the ppt $1{:}2^1_{acid}$. In the reprecipitation of precipitate 1:2, however, there was no detectable activity in the ppt $1{:}2^1_{acid}$. Another difference was observed with regard to protein. Thus, whereas 61% of the protein occurred in the ppt $1{:}2^1_{acid}$ following reprecipitation of precipitate 1:2, the reverse was true for precipitate 1:4, with 66% of the protein remaining in the final supernatant$_{acid}$ and only 19% occurring in the ppt $1{:}2^1_{acid}$.

5.15.2 SDS Gel Electrophoresis of Final Supernatants Following Reprecipitation of Precipitate 1:2 and 1:4 Solutions

SDS gel electrophoresis of the final supernatants (10 µg), obtained by reprecipitation of precipitates 1:2 and 1:4 following acidification, showed that each contained a major component with the same mobility.

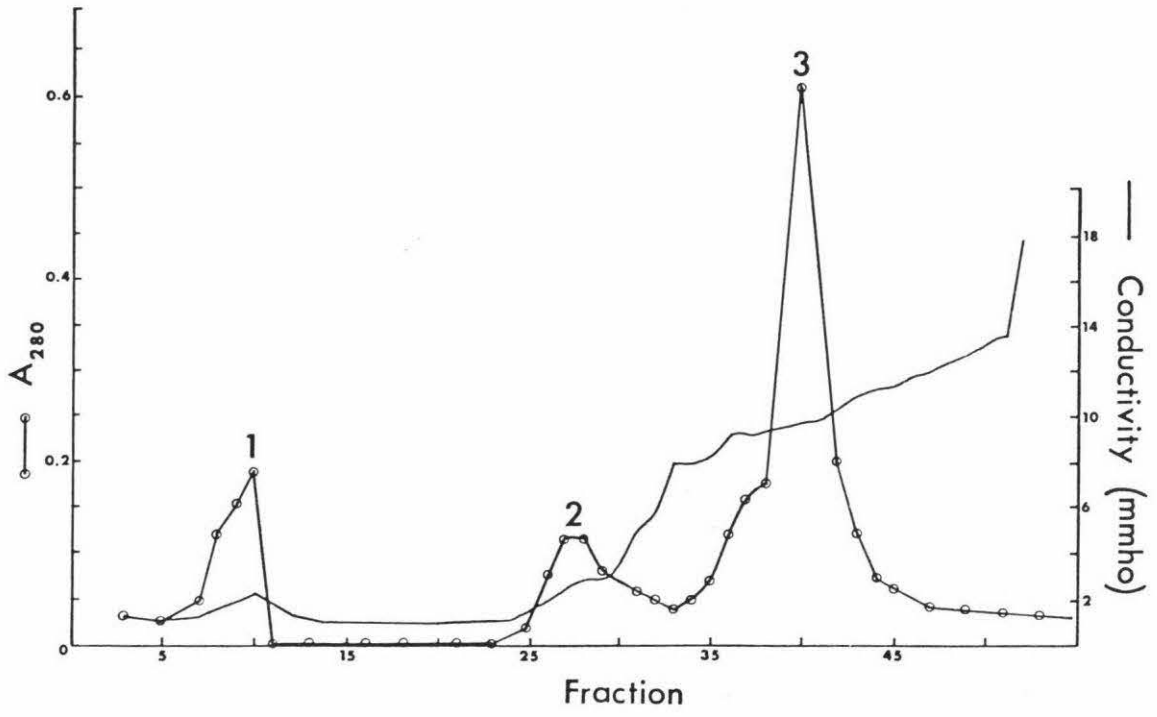
A small shoulder was also observed on the leading front of the major peak of the supernatant from ppt 1:2. SDS gel electrophoresis of the acetone precipitate (8 μg) of the final supernatant after reprecipitation of ppt 1:2 following acidification also showed a major component, and a lesser component with a greater mobility than the former.

5.16 Cellulose Phosphate Column Chromatography of BSP Preparations (Methods 3.12)

5.16.1 Dialyzed, BSP Precipitate 1:2

The elution profiles (I, II) of samples of dialyzed, BSP ppt 1:2 (2390 units/mg), fractionated on cellulose phosphate, are shown in Fig. 21A. In column I no antibacterial activity was detected in fractions 23 to 49, which were assayed directly. Therefore, 5 cm^3 samples of peak 1 (fractions 8 to 10), peak 2 (26 to 30) and peak 3 (38 to 43) were dialyzed against distilled water for 4 h to remove citrate, and then concentrated, 10, 10 and 5 times respectively, by lyophilization and resuspension in a known volume of distilled water. No antibacterial activity was detected in peaks 1 or 2. Assuming that no activity was lost upon dialysis, then 11% of the applied activity was associated with peak 3. More realistically, probably at least 40% of the activity was lost upon dialysis (see Fig.17), which would result in a recovery of 18% of the applied activity. Pooled fractions, 33 to 37 and 44 to 48, on either side of peak 3, were also concentrated as described above, but only small, very uneven zones were observed upon determination of antibacterial activity. A second citrate wash of the cellulose phosphate did not elute any additional protein. Protein concentrations were not determined, because Tris buffer interferes with the determination of protein by the Lowry method (Kuno and Kihara, 1967).

COLUMN I



COLUMN II

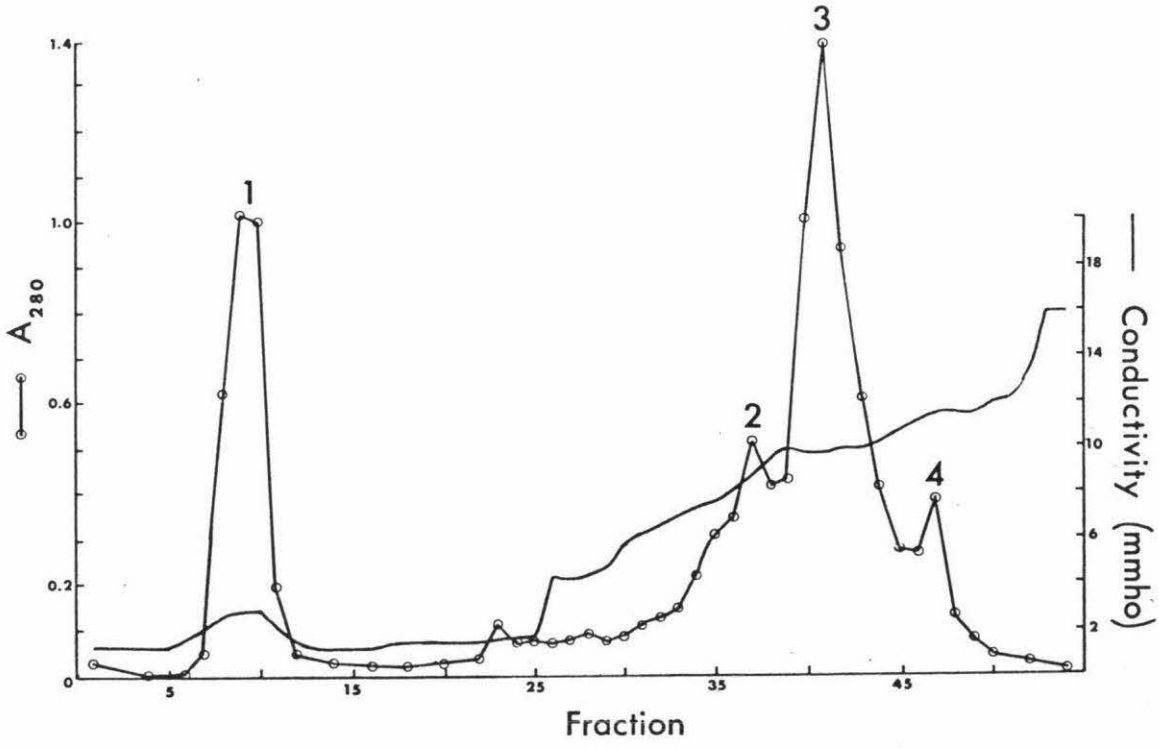


Fig. 21A Cellulose phosphate chromatography of a dialyzed, BSP 1:2 precipitate (2390 units/mg)
I : 24.8 mg protein; 59,375 antibacterial units
II : 74.4 mg protein; 174,990 antibacterial units
and elution with a sodium citrate gradient : I (0 to 0.5M); II (0.1 to 0.4M).

Three times as much dialyzed, precipitate 1:2 was applied to column II than column I. Fractions 6 to 50 were assayed directly for antibacterial activity, and 23% of the initial activity was recovered as shown in Table XXXVIII.

The low recovery of activity obtained for both columns I and II suggested that perhaps Tris buffer affected the release of activity from the cellulose phosphate. Previously, all batch experiments were conducted in phosphate buffer.

5.16.2 Cellulose Phosphate Batch Treatment of BSP and Dialyzed, BSP Precipitate 1:2

A comparison of the adsorption of activity from BSP (23,625 units/cm^3) and dialyzed, BSP ppt 1:2 (11,875 units/cm^3) to cellulose phosphate equilibrated in phosphate buffer or Tris - HCl buffer, is presented in Table XXXIX. For each treatment, the activity recovered was found entirely in the final CP eluate.

Table XXXIX. Comparison of batch extraction of antibacterial activity from dialyzed, BSP ppt 1:2 and whole BSP with cellulose phosphate equilibrated in 0.1M sodium phosphate buffer, pH 7.4, or 0.05M Tris - HCl buffer, pH 7.4

Treatment	Sample (cm^3)	CP Buffer	% Activity Recovered
	Dialyzed, ppt 1:2		
A	2.0	Phosphate	18
B	2.0	Phosphate	22
C	1.0	Tris	0
D	2.0	Tris	12
	Whole BSP		
E	2.0	Phosphate	144
F	2.0	Tris	171

Table XXXVIII. Cellulose phosphate chromatography of dialyzed, BSP ppt 1:2 (II) and BSP ppt 1:2[1] (III, IV)

Fraction	Antibacterial Units/A_{280} Unit			% of Applied Units		
	II	III	IV	II	III	IV
32	ND*	5329	ND*		1.4	
33	ND*	7483	ND*		1.9	
34	ND*	9074	ND*		2.6	
35	ND*	6682	ND*		2.6	
36	1378	9840	ND*	1.7	3.3	
37		7954	ND*		2.5	
38	1870	5615	ND*	2.2	1.8	
39	ND*	2360	ND*		0.7	
40	1058	4375	ND*	3.1	1.5	
41	734	8333	7681	2.9	3.5	3.4
42	-	10,000	10,377	-	5.5	6.3
43	1642	14,428	13,245	2.9	10.2	10.3
44	2139	15,213	12,958	2.1	14.4	11.8
45		13,458	13,239		12.7	12.0
46	3745	17,750	14,375	2.9	12.5	11.8
47	3000	18,036	14,423	3.3	8.9	12.6
48	5606	12,676	24,583	2.1	3.2	3.8
				23.2	89.2	72.0

* ND = no activity detected.

The results (E,F) indicate that the buffer in which the cellulose phosphate was equilibrated, did not affect the adsorption or release of BSP activity from cellulose phosphate; a 30% greater potentiation of activity was, however, observed with Tris buffer.

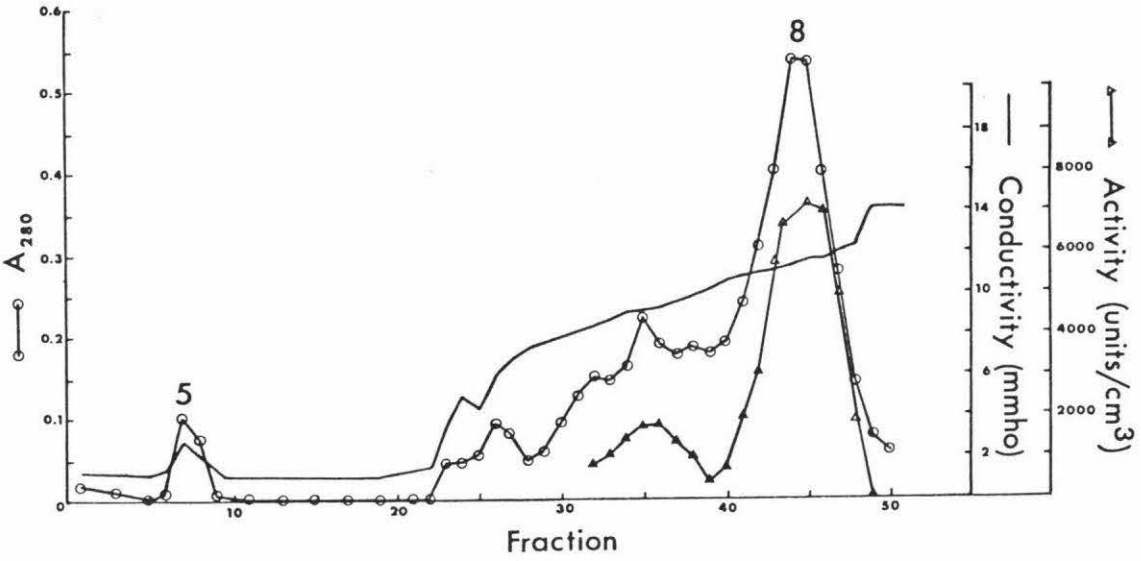
In contrast, the maximum recovery of activity for the dialyzed, BSP ppt 1:2 was only 22%. Very little difference in recovery was observed following treatment with either cellulose phosphate in Tris buffer or phosphate buffer (A,B,D). Treatment C exemplified the loss of activity observed with the dialyzed material. That is, although 11,875 units (ample for detection in the total 12 cm^3 volume) were applied to the cellulose phosphate, no activity was recovered. The recovery levels obtained in these batch experiments were similar to the low levels obtained following column chromatography of the dialyzed, BSP ppt 1:2.

5.16.3 BSP Precipitate $1:2^1$

For comparison, the elution profiles (III,IV) of two solutions of $1:2^1$ precipitates (from ethanol reprecipitation of 1:4 ppt solutions), fractionated on cellulose phosphate (III,IV), are shown in Fig. 21B. The direct assay of column III fractions showed that 89% of the applied activity was recovered. This activity was associated with fractions 32 to 48. A detailed analysis of these fractions is presented in Table XXXVIII.

Twice as much protein was applied to column IV, and three major peaks were observed. The fractions were assayed directly, and 72% of the initial activity was recovered. The activity was associated with peak 8 (fractions 41 to 48). The increase observed in antibacterial units/A_{280} units (Table XXXVIII) suggested that peak 8 was composed of two components. The greater specific activity of fractions on the right side of the peak could be due to decreased amounts of inactive or less

COLUMN III



COLUMN IV

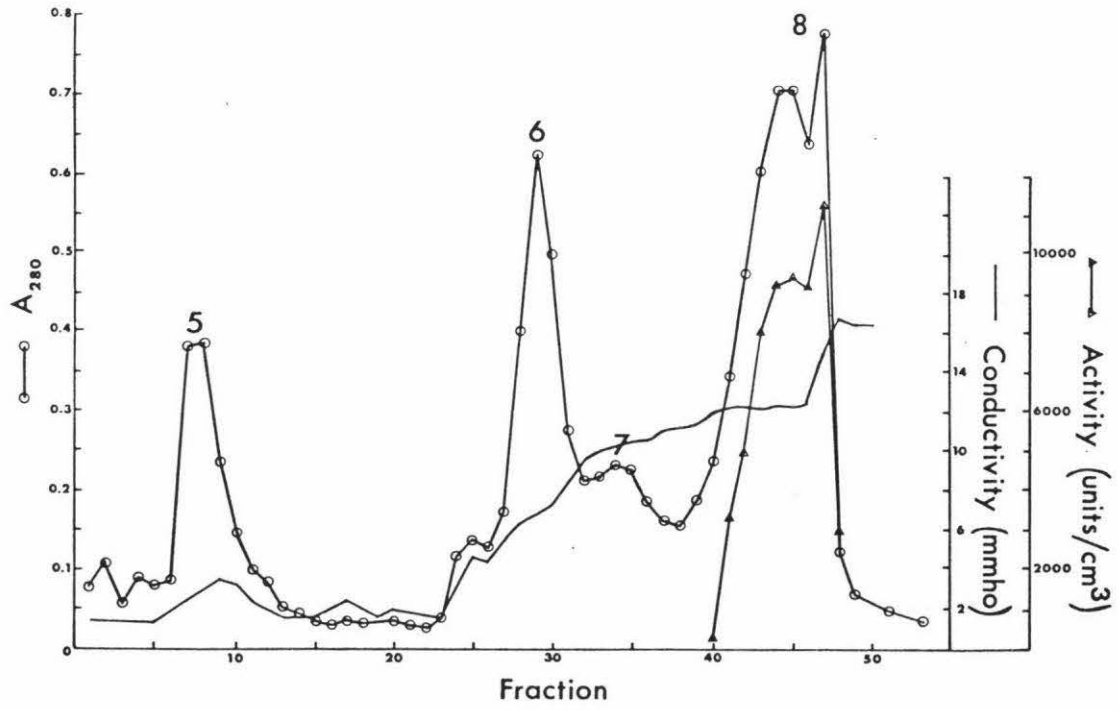


Fig. 21B Cellulose phosphate chromatography of BSP 1:2[1] precipitates: III (11,380 units/mg; 22.4 mg protein; 283,800 antibacterial units) IV (8740 units/mg; 44.6 mg protein; 390,000 antibacterial units) and elution with a citrate gradient (0.1 to 0.4M).

active protein and therefore, less contamination of the more active component. The high recovery of activity obtained for both 1:2[1] precipitates was markedly different from that obtained following fractionation of the 1:2 precipitate.

A comparison of the various peaks from each column and the conductivity at which they were eluted is presented in Table XL. Peak 7 (ppt 1:2[1], IV) and peak 3 (ppt 1:2, I and II) were eluted at about the same conductivity. Peak 8 (ppt 1:2[1], III) showed the same conductivity as peak 4 (ppt 1:2, II).

5.16.4 Calculated Specific Activity of BSP Antibacterial Factor

From the results obtained for columns III and IV, a simple calculation estimates the specific activity which could be achieved for purified BSP antibacterial factor. For example, column IV:

1) total Absorbance at 280 nm = 11.2
2) total A_{280} units of peak 8 (38-48) = 5.1 = 46% of total A_{280} units
3) since the specific activity of the starting material = 8740 units/mg and only 46% (0.46 mg) of 1.0 mg represents peak 8, then
4) specific activity of peak 8 = 8740 ÷ 0.46 = 19,000 units/mg
5) average antibacterial units/A_{280} units = 13,800
6) best fraction (48) = 24,500 antibacterial units/A_{280} unit which is 1.8 times better than the average. Therefore,
7) calculated specific activity = 19,000 x 1.8 = 34,200 units/mg.

5.17 Immunodiffusion Studies

5.17.1 Antisera Against Purified BSP and Pancreas Preparations

5.17.1.1 Rabbits A and B (BSP preparations)

Both rabbits A and B produced antibodies against the BSP preparations

Table XL. Conductivity at which various fractions (Fig. 21A, 21B) were eluted following fractionation of BSP preparations on cellulose phosphate and elution with a citrate gradient

Column	Peak	Fraction No.	Conductivity (mmho)	Column	Peak	Fraction No.	Conductivity (mmho)
I	1	8-10	2.4	III	5	7-8	2.9
	2	26-30	2.9		8	42-47	11.5
	3	38-43	10.3				
II	1	8-10	2.8	IV	5	7-9	2.9
	2	37	8.2		6	28-30	6.7
	3	40-42	9.9		7	33-35	10.2
	4	47	11.6		8	43-47	12.5

(A = ppt 1:4[1], 17,370 units/mg; B = ppt 1:3[1], 16,860 units/mg) with which they were immunized. Plate 2 illustrates the precipitin reactions which were observed between antisera A and B and various purified BSP preparations, ranging from 0.7 to 8.3 mg protein/cm^3.

Antisera A and B were tested against fifteen purified preparations, and in eight cases the same number of precipitin lines (1 to 4) was observed with either antiserum. However, with other samples, often one and sometimes two additional precipitin lines were formed with antiserum B. These results suggested that antiserum B was more heterogeneous than antiserum A. There was no correlation between the specific activity or protein concentration of a preparation and the number of precipitin lines observed, as shown in Table XLI.

Some antigens showed four precipitin lines against antiserum B. With time, the two lines which were observed closest to the antibody well seemed to coalesce. The precipitin lines were designated 1a, 1b, 2 and 3 reading away from the antibody well.

Initially, no reaction was observed between either antiserum A or B and the crude pancreas extract or final pancreas - CP eluate. However, when the antisera were tested against two different preparations of crude extract and final CP eluate a very faint reaction was observed, and with the eluate, a second faint precipitin reaction was observed further from the antibody well. The very faint reaction closest to the antibody well was also observed when antisera A and B were run against the pancreas preparations, which had been used to immunize rabbits C and D.

Antisera A and B did not produce a precipitin reaction with histones f1, f2a, f2b and f3 tested at 0.1, 0.5 and 1.0 mg/cm^3. A variation, in which the antigen well (4 mm diameter) was filled 2 h after the antibody well (5 mm diameter) to allow pre-diffusion of the larger antibody molecules, also showed no precipitin reaction.

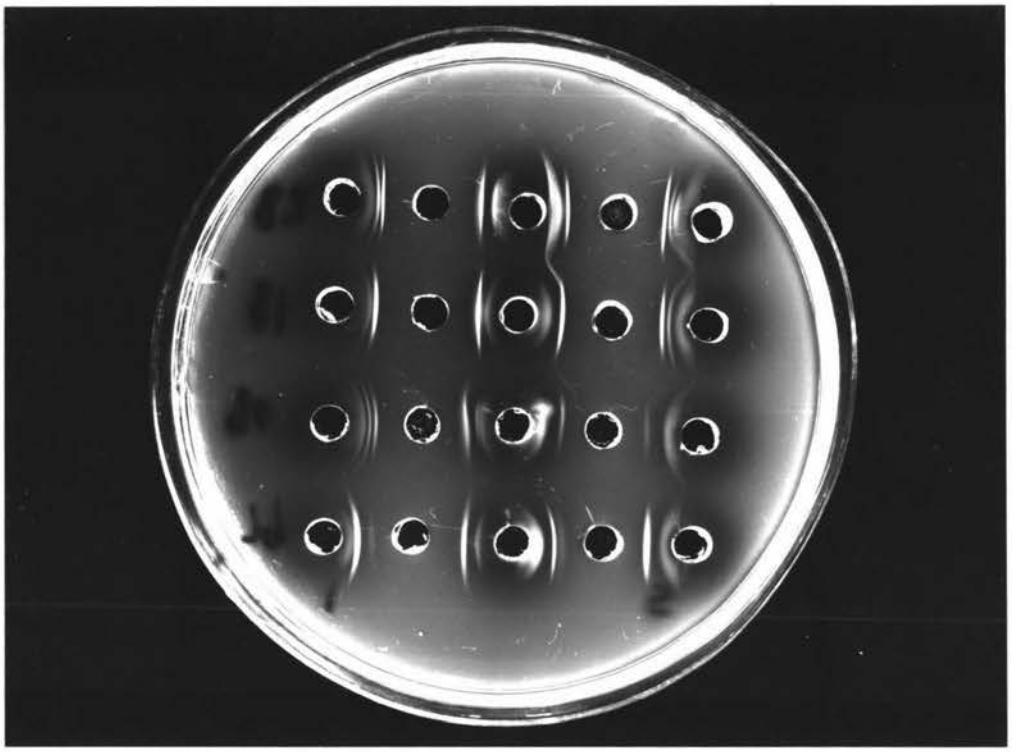


Plate 2: Immunodiffusion studies : various BSP preparations versus BSP antisera.

Note the precipitin lines between the wells.

Table XLI. Immunodiffusion studies: various BSP preparations versus BSP antisera

BSP Preparation	Specific Activity (units/mg)	Protein (mg)	No. of Precipitin Lines Formed Against Antiserum	
			A	B
1	6620	4.0	3	3
2	6770	2.4	3	3
3	7740	4.8	2	3
4	8430	8.3	3	3
5	8570	1.4	2	3
6	8740	4.5	2	3
7	8970	1.7	3	4
8	9500	6.0	3	3
9	9600	0.7	2	4
10	10,560	0.9	1	1
11	11,380	5.2	3	4
12	11,620	4.0	2	2
13	13,220	1.0	2	2
14	17,370 (A)	1.0	3	3
15	16,860 (B)	0.9	2	3

Immunodiffusion studies on (i) the final supernatant (concentrated 20 times) and the acetone precipitate of the final supernatant, from the reprecipitation of ppt 1:2 after acidification (Results 5.15.1.3), and (ii) the final supernatant of a ppt 1:4 following similar treatment (Results 5.15.1.4), showed one diffuse precipitin reaction close to the antiserum A well in all three samples. The samples from the 1:2 precipitate, however, also showed a more distinct precipitin reaction further from the antibody well. These results agree well with SDS gel electrophoresis of the final supernatants (Results 5.15.2).

5.17.1.2 Rabbits C and D (Pancreas preparations)

Rabbits C and D produced no or very little antibody against pancreas preparation 1 (19,130 units/mg). A very faint precipitin reaction was observed once (after 96 h incubation of antiserum C run against pancreas antigen (1)). A variation, in which the antisera wells were refilled after 2 h, failed to produce any observable precipitin reaction.

Following immunization of rabbits C and D with pancreas preparation 2 (11,190 units/mg), a single precipitin reaction was observed between antiserum C and pancreas antigen (2), and a very faint reaction was observed between antiserum D and pancreas antigen (2).

Antisera C and D were also tested against two BSP precipitates $1:2^1$ and $1:4^1$. Fig. 22A represents a line drawing of the precipitin reaction which was observed between antisera C and D, and BSP ppt $1:2^1$ (11,380 units/mg); no reaction was observed with BSP ppt $1:4^1$ (18,400 units/mg). These results may be a reflection of the lower protein concentration of ppt $1:4^1$ (0.9 mg/cm^3) versus the more concentrated ppt $1:2^1$ (4.5 mg/cm^3).

Further studies demonstrated that the precipitin line produced by both antisera C and D against BSP ppt $1:2^1$ shared identity with the precipitin line produced by antiserum C against pancreas antigen (2) as shown in Fig. 22B.

Fig. 22A. Immunodiffusion : pancreas antiserum versus BSP antigens

1,2 : -

3,6 : antiserum D

4 : BSP ppt $1:2^1$

5 : BSP ppt $1:4^1$

centre : antiserum C

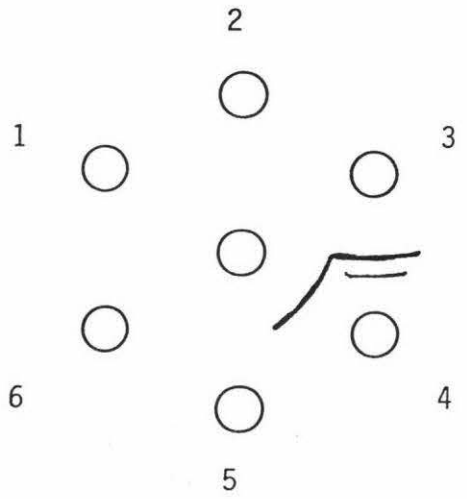


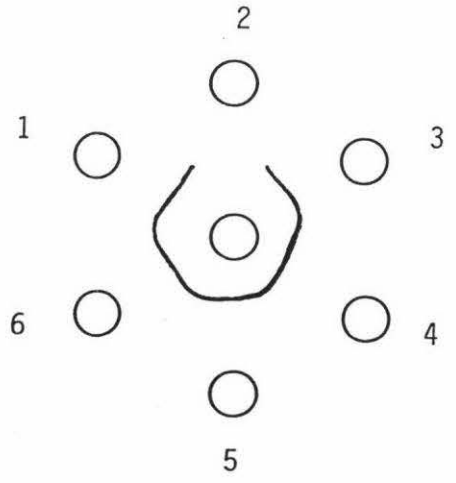
Fig. 22B. Immunodiffusion : pancreas antiserum versus pancreas antigen (2) and BSP antigens

1,3,6 : pancreas antigen (2)

2 : -

4,5 : BSP ppt $1:2^1$

centre : antiserum C



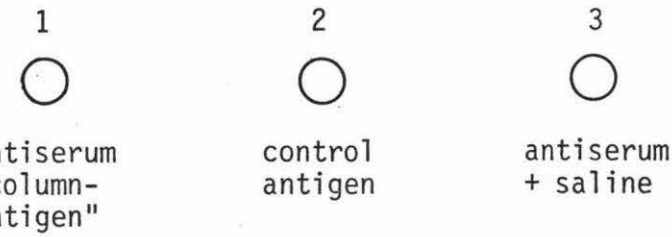
In summary, the immunodiffusion studies indicated that:

1) both BSP preparations elicited a good antibody response
2) the BSP preparations were not homogeneous but elicited a 3 to 4 component antibody response
3) only one pancreas preparation elicited a weak antibody response
4) the purified BSP preparations were not identical to the pancreas preparations since different antibody responses were obtained. However, one component in the BSP preparation seemed to elicit an antibody which would react weakly with a component in the pancreas preparation. Similarly, the pancreas antisera were shown to react with component(s) of a BSP preparation. This BSP component showed identity with a component in the pancreas preparation when these were run against antiserum C. Perhaps the antibacterial fraction of both purified preparations has limited antigenic activity, and may elicit a poor antibody response. The possibility that only contaminating proteins, and not active factor, elicit an antibody response, can not be overlooked.

5.17.2 Correlation of Chromatographic Peaks with Precipitin Lines

5.17.2.1 Absorption of Antibody by "Column-Antigen" (Methods 3.13.4.1)

In an attempt to match a given precipitin line with a particular peak ("column-antigen") from columns I to IV (Fig. 21A, 21B), rows of wells (12 mm from center to center) were punched in immunodiffusion agar and samples were tested as follows:



An antigen, identical or similar to that used for the immunization of the rabbits, was placed in well 2. This antigen generates multiple precipitin lines against BSP antiserum in well 3. A mixture of antiserum and "column-antigen" was placed in well 1.

The precipitin lines, which were produced with a control antigen versus the antiserum, were compared with those produced by the same antigen versus the antiserum absorbed with "column-antigen". The disappearance of a precipitin line was evidence for the association of that line with the "column-antigen". Initially, three different control antigens were used and the results are included in Table XLII. A distinction between components 1a and 1b could be made with only one antigen.

Precipitin reaction 1 was not well defined as were precipitin lines 2 and 3. On the contrary, it tended to be a more diffuse reaction very near the antibody well. In the absorption studies a partial disappearance of reaction 1 led, in some cases, to difficulty in matching a peak with precipitin reaction 1.

In all absorption studies a one to one ratio of antiserum to antigen was used. In some instances greater clarity might have been achieved by varying the relative amount of antigen.

The linear arrangement absorption studies were advantageous, in that the precipitin lines were clearly visible, and no interfering reactions were obtained due to lack of surrounding wells. However, some difficulties arose in determining which of the lines had disappeared. Therefore, a second well pattern was also used.

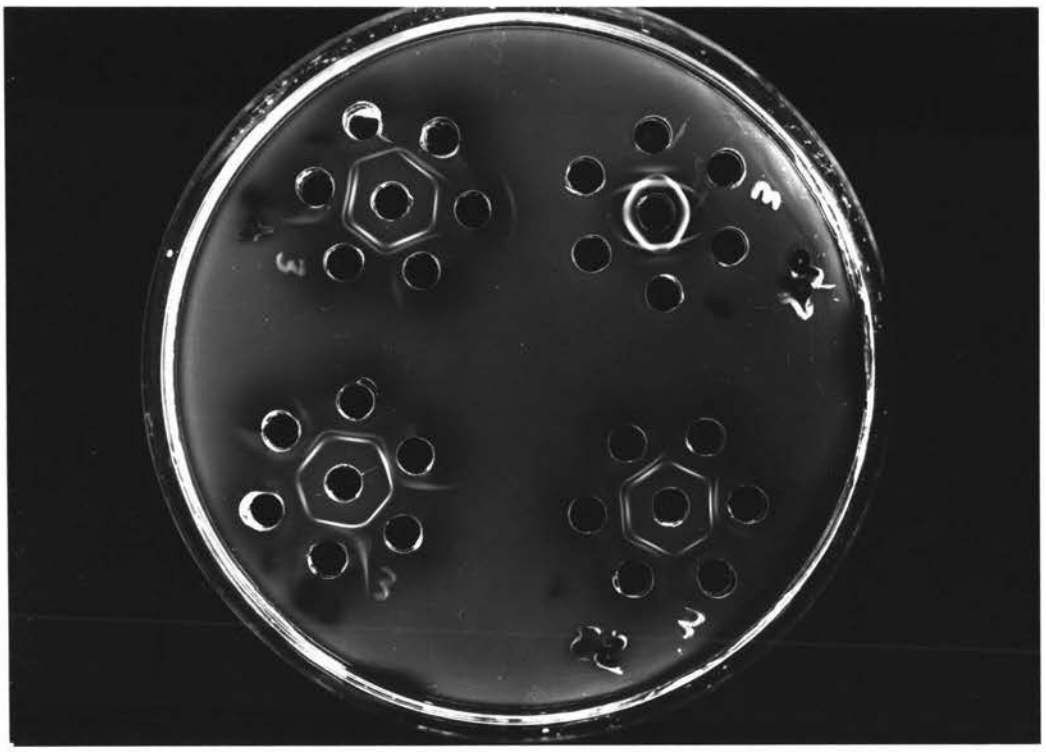
Plate 3 (upper left and lower patterns) shows typical results using this well pattern. Line drawings of 3 studies are presented in Fig. 23A, B and C.

Table XLII. Serological identification of BSP protein fractions obtained by cellulose phosphate column chromatography (see Fig. 21A, 21B for elution profiles)

Column	Peak	Precipitin Lines		
		Linear Pattern	Circular Pattern	Tentative Identification
I	1	NR*	NR*	-
	2	NR*	NR*	-
	3	2	2, some 1 (a)	2
II	1	3	3	3
	2	not tested	2, some 1	2
	3	2	2, 1 (a)	2
	4	1 (a), 2	2, 1	1 (a)
III	5	not tested	not tested	-
	8	not tested	1 (a)	1 (a)
IV	5	NR*	NR*	-
	6	1 (b)	1 (b)	1 (b)
	7	1 (a,b), 2	2, 1 (b)	2
	8	1 (a)	1 (a)	1 (a)

* no response : no visible reaction occurred between antiserum B and the "column-antigen", therefore, no absorption study was done.

A



Y B

Plate 3: Correlation of "column-antigens" with precipitin lines by antibody absorption (A, B, Y).

Fig. 23A. Line drawing of Plate 3A (upper left). Fig. 23A shows that peak 1 (II) absorbed the antibody which reacts with the control antigen to form precipitin line 3. Similarly, peak 4 (II) may be matched with line 2, and peak 6 (IV) may be matched with precipitin reaction 1.

2,4,6 : antiserum B

1 : antiserum B + peak 1 (II)

3 : antiserum B + peak 4 (II)

5 : antiserum B + peak 6 (IV)

centre : control antigen

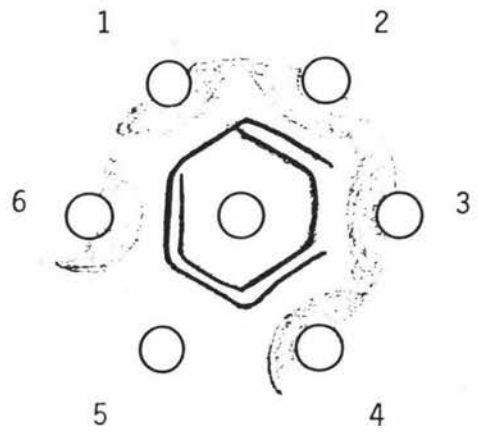


Fig. 23B. Line drawing of Plate 3B (lower right). Fig. 23B shows that peak 7 (IV) absorbed antibodies which react with the control antigen to form lines 1 and 2. Peak 2 (II) was equated with line 2, and the fraction in well 6 did not absorb any antibody at the concentration used.

1,3,5 : antiserum B

2 : antiserum B + peak 7 (IV)

4 : antiserum B + peak 2 (II)

6 : antiserum B + peak 23 (II)

centre : control antigen

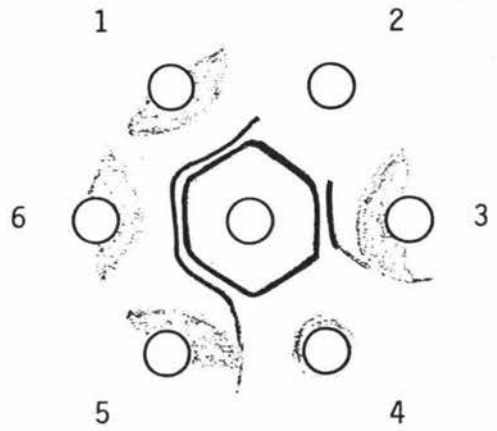


Fig. 23C. Line drawing of immunodiffusion study for the correlation of "column-antigens" with precipitin lines. Fig. 23C shows the 4 component system clearly. Thus, both peaks 3 (I, II) were matched with components 1a and 2. Peak 8 (IV) absorbed the antibody which reacted with the control antigen to form reaction 1a.

2,4,6 : antiserum B

1 : antiserum + peak 3 (I)

3 : antiserum + peak 8 (IV)

5 : antiserum + peak 3 (II)

centre : control antigen

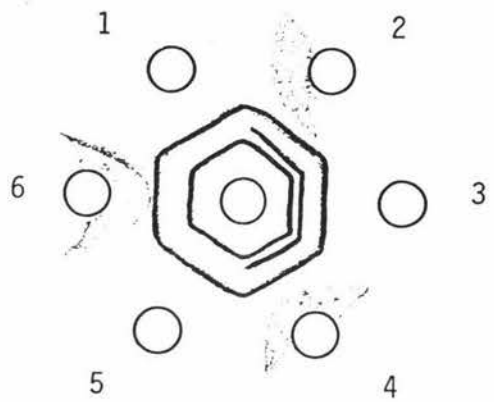


Fig. 24. Immunodiffusion : identification of chromatographic peaks

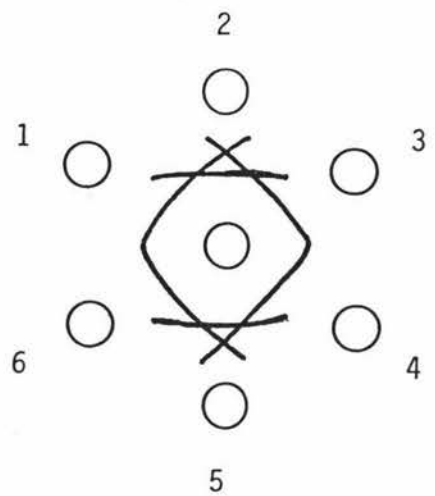
1,6 : peak 6 (IV)

2,5 : peak 8 (IV)

3 : peak 3 (II)

4 : peak 3 (I)

centre : antiserum B



With the second system, a given line could be identified more easily, since the formation of a continuous line with adjacent precipitin lines produced by unabsorbed antibody provided evidence for identity. The antiserum-antigen mixtures were tested against 3 control antigens and a summary of the results is included in Table XLII.

Finally, aliquots of several peaks were run in adjacent wells against antiserum B in an attempt to show identity (continuous line) or non-identity (intersecting lines) of one peak with another. For example, Fig. 24 shows that peak 6 (IV) and peak 8 (IV) are not the same. The figure also shows that peak 3 (I) is the same as peak 3 (II). In similar studies, peak 8 (III) and 8 (IV) showed identity, and peak 4 (II) showed identity with peak 8 (IV) and non-identity with peak 6 (IV).

In relating the various chromatographic peaks to each other, and to particular precipitin reactions, the preceeding results were summarized, and the following conclusions were made.

Previously, it was shown that antibacterial activity was associated with peak 3 (I), peaks 2, 3 and 4 (II), peak 8 (III) and peak 8 (IV). The immunodiffusion results showed that each of the peaks could be related to precipitin line 1, and more specifically component 1a. Such results provide a partial explanation for the greater recovery of activity obtained from column II (active peak 4) than column I. However, peak 3 (I) and peaks 2, 3 and 4 (II) differed from peaks 8 (III) and 8 (IV) in that they were also equated with precipitin line 2.

Previously, it was shown that peaks 3 (I) and 3 (II) showed the same conductivity as peak 7 (IV), and it was suggested that perhaps they were the same. All three peaks were related to reactions 1a and 2, but peak 7 (IV) was also matched with precipitin line 1b. The 1b component of peak 7 (IV) may be a carry-over from peak 6 (IV), which was equivalent to

component 1b and showed no antibacterial activity; the 1a component may be due to contamination with peak 8 (IV). Consequently, 7 (IV) is probably equivalent to precipitin line 2. Peak 6 (IV) was not related to any peak from columns I, II or III. Finally, peak 1 (II) was related to precipitin line 3.

5.18 Neutralization of Antibacterial Activity by Antisera

Preliminary attempts (Methods 3.13.5) failed to demonstrate neutralization of the antibacterial activity of purified BSP factor by antisera.

The neutralization of two different, purified BSP preparations by antisera A and B, was attempted using the broth dilution technique. However, neutralization of the antibacterial activity could not be determined, due to the antibacterial effect exhibited by both the normal rabbit serum and the two antisera alone. In a plate diffusion assay, a 1:20 dilution of the rabbit sera was sufficient to eliminate this serum effect.

Histones f1 and f2a and a BSP ppt $1:4^1$ (10,560 units/mg) were each mixed with antiserum to yield a final antiserum concentration of 1:20. Although, at this concentration, no antibacterial effect of antiserum was observed by the plate diffusion assay, the activities of mixtures of antiserum and histone f2a, and of antiserum and BSP ppt $1:4^1$ were enhanced, as indicated by increases in zone sizes over those observed for appropriately diluted samples alone. The antibacterial activity of histone f1, on the other hand, was decreased 33% and 30% in the presence of antisera A and B respectively. This decrease may represent a non-specific serum effect.

DISCUSSION

DISCUSSION

1. Isolation of Basic Antibacterial Proteins from Animal Tissues and Fluids

A number of cationic proteins which have been isolated from animal tissues and fluids have an antimicrobial effect against a variety of bacteria, including E. coli, S. typhimurium, P. aeruginosa, K. pneumoniae, streptococci and staphylococci (Hirsch, 1958; Zeya and Spitznagel, 1966b; Hibbitt et al., 1969). The isolation and characterization of these basic proteins have been of considerable interest in attaining a better understanding of the natural defence systems of animals.

Although initial studies involved the extraction of these substances from homogenized animal tissues, such as thymus and pancreas (Bloom et al., 1947; Dubos and Hirsch, 1954; Skarnes and Watson, 1956a), more recently, cationic proteins with antimicrobial activity have been isolated from secretions such as bovine cervical mucus (Brownlie and Hibbitt, 1972), prostatic fluid (Stamey et al., 1968) and bovine seminal plasma (Shannon et al., 1974, 1975). This study involved the isolation, purification and characterization of antibacterial factors from bovine seminal plasma and, for comparison, from bovine pancreas.

Most of the methods of extraction of basic proteins from tissues and secretions have involved the use of acid to dissociate the cationic material from other components of the tissue, because of the acid solubility of the basic proteins. For example, the basic histones are generally prepared by the acid extraction of nuclei or nucleoprotein with 0.1 to 0.3N HCl or H_2SO_4 (Phillips, 1962). Other basic antibacterial proteins have been isolated from polymorphonuclear leukocytes (Zeya and Sptiznagel, 1966b), calf thymus (Hirsch and Dubos, 1954), bovine teat canal keratin (Hibbit and

Cole, 1968), bovine milk cells (Hibbitt et al., 1971) and a variety of other tissues (Bloom et al., 1947),by acid extraction, often for 2 to 4 days at room temperature. This extraction procedure may lead to the acid hydrolysis of certain proteins, and consequently, the basic peptides may be products of hydrolysis, rather than natural tissue constituents (Dubos and Hirsch, 1954).

Phillips (1962) states that the chief causes of degradation are the acid and alkaline proteinases; he suggests that any preparative procedure must aim to avoid the action of these widespread enzymes. In achieving this aim, low temperature and rapidity of preparation are essential (Kincade and Cole, 1966). Consequently, the optimal conditions for the extraction of antibacterial activity from bovine pancreas were determined (Results 3.1). The standard procedure involved cutting the tissue while partially frozen, homogenizing it in the presence of chilled, 0.2M citrate solution, pH 2.0, and heating the mixture promptly at 65^{o}. Both acid and heat were used to destroy the proteolytic enzymes of the tissue, thereby reducing the production of breakdown products.

Milder processes have been employed for the extraction of basic proteins. Brownlie and Hibbitt (1972) isolated cationic proteins from cervical mucus by column chromatography on carboxymethyl cellulose. In this study, a mild procedure for the extraction of cationic proteins from bovine seminal plasma was developed.

Initially, the basic BSP proteins were isolated by adsorption to DNA, an anionic polymer with negatively charged phosphate groups; this was followed by elution with sodium citrate. Due to the poor recovery of antibacterial activity by this method, an alternative procedure was devised.

Cellulose phosphate (CP), a cation exchange resin which binds basic residues strongly (Dawson et al., 1972), was found to be effective in adsorbing proteins from BSP and pancreas extracts. The use of cellulose phosphate for the extraction of basic proteins is an especially mild

procedure, compared to many of the procedures outlined in the literature.

Previous work (Schollum, 1977) on ion exchange column chromatography of bovine seminal plasma showed the elution of the antibacterial activity as a broad, non-distinct profile. Consequently, initial experiments using cellulose phosphate involved batch separations.

Although less efficient than column techniques, batch methods are advantageous in that they are rapid and technically easier because: (i) no difficulties arise due to swelling or shrinking of the resin and (ii) desorption of a substance can be easily accomplished by resuspending the slurry in solutions of higher ionic strength or modified pH (Pharmacia, 1976). Sodium chloride (0.2M) was used to elute contaminating, non-active proteins, as well as some active proteins, from the cellulose phosphate. The bulk of the active protein was eluted from the cellulose phosphate with 1.6M sodium citrate.

The use of CP-batch separations involved the determination of the optimal conditions for the recovery of antibacterial activity from BSP or pancreas extracts. Variations in the pH of the CP slurry, the ratio of CP to crude material, and the concentration and volume of sodium chloride and sodium citrate washes, were studied, and a standard isolation procedure was devised (Methods 2.3.3.1).

The procedure outlined for seminal plasma and pancreas extracts was the same, except for the use of different amounts of cellulose phosphate with the crude material. Whereas a ratio of cellulose phosphate to crude material of 1.2 was used for BSP, a ratio of 0.2 was used for pancreas extracts. Although the lower amount of cellulose phosphate used with the pancreas extract results in a 30% loss of antibacterial activity, a much better preparation with respect to specific activity is obtained than when a ratio of 1:2 is used. In contrast, a low ratio of CP to BSP (0.2) results in a loss of 55 to 60% of the activity, with no marked increase in specific

activity. The different results are probably a reflection of the different protein concentrations of the two materials - BSP may contain 100 mg protein/cm^3, whereas pancreas extracts often contain approximately ten times less protein (10 to 16 mg/cm^3).

As reported previously, an increase in activity (approximately 70% over the initial detectable activity of BSP, was observed following cellulose phosphate treatment. No increase was observed following the treatment of pancreas extract. The increase may be due to the removal of a protein which directly inhibits the activity of the active fraction. Or, more likely, the treatment may remove proteins which non-specifically mask the active protein, by inhibiting its diffusion in the agar diffusion assay. That is, whereas whole seminal plasma may contain 100 mg protein/cm^3, final CP eluates usually contain only 3 mg/cm^3. Similarly, crude pancreas extracts have a much lower protein concentration (13 mg/cm^3) than BSP. Thus, this masking phenomenon may not occur, and an increase in initial activity would not be observed with pancreas extracts.

This proposed masking effect could explain the non-linear response observed upon dilution of whole BSP (Fig. 7). Perhaps dilution dissociates the active protein from masking proteins, thereby allowing it to diffuse further in the agar with an observed increase in zone size and antibacterial activity.

That the basic antibacterial proteins adsorb to the cellulose phosphate due to a simple electrostatic interaction, rather than an affinity for phosphate groups, was shown by a comparison of the elution of the proteins from CP with sodium phosphate, sodium chloride and sodium citrate (Table XV). As no activity was eluted with sodium phosphate of a comparable ionic strength to sodium chloride, which eluted 40% of the recovered activity, the proteins did not show a specific affinity for phosphate groups.

Following extraction of basic proteins from animal tissues, ethanol is commonly used as a convenient and effective agent for the precipitation of

the isolated proteins.

A comparison of ethanol precipitations of a BSP-CP eluate at 0^{o} and 25^{o} showed that protein solubility, in the presence of ethanol, decreased markedly at the lower temperature, necessitating adequate temperature control during each fractionation. The pH must also be controlled for reproducible results, because a protein which is soluble at a given pH, may be precipitated almost completely at another pH (Green and Hughes, 1955). All standard ethanol precipitations were, therefore, done in 0.01M sodium phosphate buffer, pH 7.4.

A comparison of ethanol precipitations of final CP eluates from BSP and pancreas extracts showed obvious differences. Although about 50% of the BSP activity was insoluble at 67% ethanol (Table XXV), the material with the highest specific activity occurred in the 67 to 80% ethanol cut (ppt 1:2 to 1:4). Approximately 70% of the pancreas activity was insoluble at 60% ethanol (ppt 1:1.5) (Table XXVII); material with the highest specific activity was precipitated between 50 to 67% ethanol in standard preparations.

The protein concentrations of the CP eluates were not adjusted before ethanol precipitation. Whereas BSP-CP eluates usually contained about 3 mg protein/cm^3, pancreas-CP eluates were approximately ten times less concentrated. This difference in protein concentration could explain the differences in precipitation profiles. Or, the differences in precipitation could be due to the presence of a greater number of contaminating proteins in the BSP eluate (9000 units/mg protein), as compared to the pancreas eluate (13,000 units/mg protein). On the other hand, the different profiles might be due to two different active proteins, or the same protein modified by the extraction procedure. Perhaps the initial acid treatment of the pancreas tissue dissociates the factor from other proteins or from citrate, which would be non-ionic under acidic conditions. These modifications may change the ethanol solubility of the pancreas factor.

The ammonium sulphate fractionation of CP eluates from BSP and pancreas extracts also differed. Whereas most of the active BSP material precipitated between 40 to 70% $(NH_4)_2SO_4$ saturation, the active pancreas material was largely soluble even at 70% saturation (Table XXIX). For comparison, the ammonium sulphate concentration could have been increased further to determine the saturation at which the active pancreas protein precipitated.

Again, the fractionation may have been influenced by protein concentrations and sample purity. It is likely, however, that the pancreas and seminal plasma antibacterial activities are due to two different proteins, with different solubilities in ammonium sulphate solutions.

2. Biological Properties of Antibacterial Factors from Bovine Seminal Plasma and Pancreas

The broth dilution assay, for the determination of the minimal inhibitory concentration of a given preparation, was used to compare the potency of the natural bovine polypeptides with that of a synthetic basic polypeptide, polylysine. Whereas a pancreas preparation appeared to have about the same MIC as polylysine in nutrient broth, the average MIC of the BSP preparations (Table XXXV) was about two times higher (18 $\mu g/cm^3$), although some BSP preparations showed similar minimal inhibitory concentrations to that of polylysine.

The best BSP preparation used for the determination of the MIC of the antibacterial factor, had a specific activity of about 14,000 units/mg protein. It is feasible that the specific activity of a purified BSP preparation could be as high as 34,000, as calculated in Results 5.16.4. Then, using the constant determined in Table XXXV (MIC = $1.98 \times 10^5 \div$ specific activity), which relates specific activity to MIC, the calculated MIC would be

approximately 6 $\mu g/cm^3$. Considering the level of streptomycin at which many microorganisms are sensitive (4 $\mu g/cm^3$) (Ericsson and Sherris, 1971), and the low molecular weight of this antibiotic (582 daltons) (Grove and Randall, 1955), the basic polypeptides from seminal plasma and pancreas, which are approximately three times larger, could be considered quite potent antimicrobial agents.

Many investigators have shown that the medium composition can greatly influence the results of sensitivity tests (Ericsson and Sherris, 1971; Garrod and Waterworth, 1969; Hoeprich and Finn, 1971). Fair et al. (1973) showed that prostatic antibacterial factor (PAF) was inhibited by the addition of one part of trypticase soy broth to four parts of PAF, and that nutrient broth, beef peptone broth and other media were also inhibitory. Similarly, the addition of brain heart infusion broth or casein hydrolysate to media, markedly reduced the activity of a basic peptide isolated from calf thymus (Dubos and Hirsch, 1954). The antibacterial activity of the BSP factor was also decreased in the presence of more complex media (Results 5.6.3), probably due to the charged nature of the antibacterial substance.

The antimicrobial spectrum of whole bovine seminal plasma was determined by Schollum et al. (1977). It had marked antimicrobial activity against a wide variety of Gram positive and Gram negative bacteria as well as several mycoplasma species.

Schollum et al. (1977) observed that the antimicrobial activity of the BSP proteins does not depend on a carbohydrate moiety,as periodate treatment does not affect their activity. They also showed that the activity spectrum of BSP differed significantly from antimicrobial substances such as spermidine and lysozyme.

In this study, neither BSP nor pancreas antibacterial activity could be attributed to lysozyme activity, due to the inability of either preparation

to hydrolyze a synthetic lysozyme substrate. Also, although lysozyme is resistant to the proteolytic effect of trypsin (Padgett and Hirsch, 1967), both BSP and pancreas antibacterial factors were trypsin-sensitive.

Preliminary studies on the mode of action of the seminal plasma factor suggested that it acted on the bacterial membrane, causing leakage of protoplasmic contents (Schollum, 1977). Shannon et al. (1974) suggested that the antimicrobial effects of seminal plasma were caused by the same agent as that which reduced spermatozoal "livability", presumably by causing membrane damage. A consideration of these results led to the proposal that the antibacterial agents under study, may be phospholipases, which could act upon membrane phospholipids. Phospholipases have been demonstrated in ram and bull semen (Scott and Dawson, 1964); they cause a loss of spermatozoal phospholipids during the passage of spermatozoa through the genital tract (Poulos et al., 1973). When compared, however, to phospholipases (Hayaishi, 1955; Rimon and Shapeiro, 1959; Magee and Uthe, 1969; De Wolf et al., 1976), the properties of the isolated factors including their lack of activity on egg yolk agar (Results 5.9), suggested that the antibacterial factors isolated from bovine seminal plasma and pancreas were not phospholipases.

Due to the constant yield of the antibacterial agent isolated from different batches of BSP, it is unlikely that the active substance is from the family of bacteriocins, antibacterial substances produced by bacteria. Unlike the agents under study, most bacteriocins are non-dialyzable (Reeves, 1972), although dialyzable forms have been isolated (Krämer and Brandis, 1975; Tagg et al., 1975).

3. Physico-Chemical Properties of Antibacterial Factors from Bovine Seminal Plasma and Pancreas

3.1 General Characteristics

The antibacterial factors isolated from BSP and pancreas were heat stable and dialyzable at neutral pH. In contrast, Shannon et al. (1974) reported that the antibacterial factor which they isolated from BSP was dialyzable at pH 3.0 but not at pH 7.0. Schollum et al. (1977) found that the inhibitory activity of BSP appeared to occur as non-dialyzable complexes, in equilibrium with smaller, free-moving moieties (pH 2.5). Upon removal of these smaller forms by dialysis, a similar proportion dissociated from the higher molecular weight complexes.

Although experiments with purified BSP material showed an equivalent loss of protein and activity following dialysis, in several cases, pancreas preparations showed the loss of some protein, but a much greater loss of activity. Activation of pancreas factor by citrate, with loss of activity upon dialysis of the citrate, failed to explain the pancreas results, because after 4 and 6 hours of dialysis the activity remained the same, but the citrate concentration decreased.

The active factor might be a subunit of a more complex aggregate as suggested by Shannon et al. (1974, 1975). It may, therefore, dissociate during dialysis and the small active subunit may pass through the dialysis membrane. Another explanation, however, involving the dialysis of the active protein and the retention of contaminating proteins within the dialysis bag, seems more likely in the light of column chromatography of BSP preparations, in which only a portion of the total protein showed antibacterial activity.

The antibacterial activity of most of the basic tissue peptides, which have been isolated by different investigators, is inactiviated by anionic

polymers such as deoxyribonucleic acid. Although differences in assay systems make comparisons difficult, at DNA concentrations similar to those reported in the literature, little or no inactivation of the antibacterial activity of BSP and pancreas factors occurred. This may be important with regard to the *in vivo* action of the peptides and their role in the natural defence of the animal host.

The molecular weight of the antibacterial factors from both BSP and pancreas was approximately 15,000 daltons (Results 5.2) as determined by SDS gel electrophoresis. Sodium dodecyl sulphate dissociates proteins into their constituent polypeptide chains complexed with SDS (Weber *et al.*, 1972). As the SDS-protein complexes are negatively charged, electrophoresis on SDS polyacrylamide gels resolves polypeptide chains according to their molecular weights, and is virtually independent of protein charge.

Although SDS gel electrophoresis for molecular weight determination is an easily applied method, the technique is not without limitations. Thus, small polypeptide chains (molecular weight less than 15,000), such as cytochrome c (Fig. 18), behave abnormally. Also, protein molecules with exceptionally high charges may show deviations from the typical molecular weight versus mobility relationships (Weber *et al.*, 1972). Histones, for example, with their very high net positive charge, show atypically low electrophoretic mobilities (Weber *et al.*, 1972), and the estimated molecular weight would, therefore, be greater than the true molecular weight.

Histone H4 (10,500 daltons) from germinating pea embryos showed less mobility than the larger histones, H2A and H2B, with molecular weights of 15,000 and 13,500 respectively (Grellet et al., 1977). With these discrepancies in mind, the molecular weight estimation for the basic BSP and pancreas factors could be slightly higher than the true molecular weights.

Molecular weight was not estimated by gel filtration on Sephadex

(Whitaker, 1963) as this method assumes that the material behaves as a spherical protein; a highly charged molecule, which may exhibit a high degree of asymmetry in solution, would, therefore, cause appreciable errors in the estimation (Forrester et al., 1969).

Many of the basic antibacterial proteins from animal tissues and fluids appear to be small polypeptides with molecular weights of approximately 14,000 to 15,000 daltons - lysozyme (14,300) (Weber et al., 1972); histones H2a (14,004), H2b (13,774) and H3 (15,324) (Elgin and Weintraub, 1975). The antibacterial factors isolated from bovine seminal plasma seem to be histone-like, having a lysine to arginine ratio of 1.27, very similar to that of histone H2a (1.17) (Table XXXIII).

The most fundamental, widely used criterion for classification of the histones is amino acid composition (Elgin and Weintraub, 1975). A comparison of the amino acid composition of a BSP preparation with histone compositions (Table XXXIV) demonstrates that the BSP factor is different from any of the bovine histones reported in the literature.

Although immunological studies showed that the BSP preparation, on which the amino acid analyses were performed, was heterogeneous, SDS gel scans (one component) of the preparation suggested that the contaminating proteins: (i) have molecular sizes similar to the active factor and are, therefore, not resolved by SDS gel electrophoresis or (ii) are present only in very low, undetected amounts, and if so, then the amino acid composition is probably fairly representative of the antibacterial protein.

Unlike the histones, which are unstable in neutral phosphate buffer (Hirsch, 1958), the activity of BSP preparations does not seem to be reduced in the presence of phosphate buffer. Hirsch suggested that the decrease in bactericidal action of the histones may be due to aggregation, attack by contaminating proteases or adsorption.

With regard to antibacterial activity, the BSP and pancreas agents are more active and have higher specific activities than any of the histones tested (Table III).

One problem, which has not been solved, is that of the relative dye binding capacities of seminal plasma proteins of the bull, ram, rabbit and boar (Bennett, 1965). Although there is some degree of variation in the efficiency of dye binding to various proteins (Bradford, 1976), most proteins studied by Bradford did not differ greatly in their dye binding capacities.

The basic antibacterial factors isolated from seminal plasma and pancreas, however, behave atypically; both bind the dye, Coomassie Blue G-250, much less efficiently than other proteins. In fact, protein concentrations determined by the dye binding method were approximately 50% lower than those determined by the Lowry method. Thus, although Tris buffer does not interfere with protein determination by the protein-dye binding method (Bradford, 1976), the assay was not used to monitor the protein concentrations in fractions containing Tris buffer.

3.2 Comparison of Ethanol Precipitated Fractions from Bovine Seminal Plasma

Ethanol precipitation profiles of BSP-CP eluates, which had an initial precipitation at or below 71% ethanol (ppt 1:2.5), seemed to suggest that active material precipitated at two different ethanol concentrations. These observations led to the suggestions that: (i) two different proteins, both with antibacterial properties, are present in the cellulose phosphate eluates, (ii) there is one protein, which exists in two different forms, that is, the active protein may exist in an unbound form, as well as an associated form bound to other proteins, and may, therefore, have two different ethanol solubilities or (iii) the active factor coprecipitates

with citrate in the early stages of ethanol precipitation, and precipitates independently at higher ethanol concentrations.

Experiments involving the ethanol reprecipitation of aqueous solutions of BSP 1:2 and 1:4 precipitates, following acidification in the presence of citrate and then neutralization, demonstrated that different profiles were obtained for each fraction (Table XXVII). The general trend in the reprecipitation of precipitates 1:2 and 1:4 was, however, similar.

That is, the active protein in ppt 1:2 remained largely soluble at 80% ethanol, as did much of the 1:4 material. A good portion (36%) of the active 1:4 protein was, however, insoluble at 67% ethanol. Under normal precipitation conditions almost all of the active protein is insoluble at 80% ethanol. SDS gel electrophoresis of the final supernatants, obtained following reprecipitation of 1:2 and 1:4 precipitate solutions, showed that both contained a major component with the same mobility. The acidification process may have dissociated the active protein from other proteins or from citrate, thereby making it more soluble in ethanol.

The amount of activity recovered following reprecipitation, differed for the two precipitates. That is, 25% more activity was recovered following reprecipitation of ppt 1:4, than reprecipitation of ppt 1:2. In both cases, however, 80 to 90% of the protein was recovered which suggested that: (i) the activity was unstable in the presence of ethanol or (ii) the active protein was modified and consequently less active or apparently less active (i.e. decreased diffusion in the agar diffusion assay) under the conditions of reprecipitation.

In conclusion, the antibacterial activity present in different BSP ethanol fractions is most probably due to one active protein. However, due to differences in amounts and kinds of protein present (i.e. more contaminating proteins in ppt 1:2), the behaviour of the active protein

during ethanol reprecipitation following acidification, was modified.

A further comparison of the two BSP antibacterial fractions - ppt 1:2 and ppt 1:4 ($1:2^1$) was made following ion exchange chromatography of each on cellulose phosphate. Although different profiles were obtained, the active fraction from each preparation eluted largely at conductivities greater than 11 mmho, which suggested that the two fractions contain the same antibacterial substance. This observation may be useful in purification of the antibacterial factor in future studies.

Antisera absorption studies were used to relate the peaks of one preparation to those of another, and differences in the kinds of proteins present in precipitates 1:2 and 1:4 were noted as discussed previously (Results 5.17.2.1).

One very striking difference between the two preparations was in the total amount of activity recovered. Whereas only 20% of the initial applied activity of ppt 1:2 was recovered, approximately 80% of the initial applied activity of ppt 1:4 ($1:2^1$) was recovered. A comparison of whole BSP with ppt 1:2 following CP batch separation confirmed the low recoveries of the latter. Thus, the behaviour of the active fraction of ppt 1:2 in the presence of cellulose phosphate differed from that of ppt 1:4. Perhaps the ppt 1:2 factor was modified during dialysis of the preparation, or during the chromatographic separation, such that the active material was less stable, or the diffusion of the factor in agar was decreased and its true activity underestimated.

In this study, dialysis was used to remove the high amounts of citrate present in ppt 1:2, which may have interfered with adsorption to cellulose phosphate; ppt 1:4, having a much lower citrate concentration, was not dialyzed.

3.3 "Purified" Bovine Seminal Plasma Preparations

Although the BSP preparations used to immunize rabbits A and B appeared to consist of one component by SDS gel electrophoresis, multicomponent antisera were produced.

The problems associated with attainment of antisera are two-fold. Thus, even in a highly purified preparation, antibodies may form against trace contaminants which are not detected by conventional techniques such as SDS gel electrophoresis, and secondly, the purification process may modify molecules, resulting in changes or loss of antigenic determinants. For a given preparation, the antigen which elicits the heaviest precipitin reaction, is not necessarily the main component of the preparation. It may, however, be a better antigen, and therefore, induce a better antibody response in the rabbit, than does another component which is present at a higher concentration.

The heterogeneity of "purified" BSP material (ppt $1:2^1$) was also demonstrated by column chromatography on cellulose phosphate, in which three major protein peaks were obtained (Fig. 21B), only one of which possessed antibacterial activity. The active material seemed to be associated with antiserum reaction 1a (Results 5.17.2.1), a weak, indistinct precipitin reaction. It may be hypothesized that the active peak consists of two components - one active, the other inactive. Perhaps the diffuse precipitin reaction is due to the inactive protein; the active protein may not have elicited an antibody response at all.

Continued studies on the chromatographic separation of BSP preparations would be of value in the further purification of the antibacterial factor. Variations in column size, elution gradient, flow rate and other conditions might be useful in obtaining even better separation of the antibacterial agent from contaminating proteins.

3.4 Further Comparison of Bovine Seminal Plasma and Pancreas Antibacterial Preparations

Differences in ethanol precipitation profiles of pancreas and seminal plasma preparations were discussed previously (Discussion 1). Also, the specific activities of pancreas-CP eluates were much higher than those of BSP-CP eluates. The initial acid- citrate- heat treatment of the pancreas tissue may have disaggregated the factor, thereby increasing its effective antibacterial sites or making it more readily diffusable in the agar diffusion assay. In either case, larger inhibition zones (i.e. greater activity) would be observed.

In an effort to obtain BSP preparations with higher specific activities, whole seminal plasma was acid- citrate- heat treated (Methods 2.3.2.1.5), and the extraction of the antibacterial fraction with cellulose phosphate was compared to that of pancreas extracts. However, specific activities comparable to those of pancreas eluates were not obtained for seminal plasma. This suggested that perhaps the BSP and pancreas antibacterial activities were different.

A further comparison involving: (i) ethanol precipitation of a pancreas eluate, which had been obtained from CP treatment of crude extract prepared under acidic-citrate conditions and (ii) ethanol reprecipitation of solutions of BSP precipitates 1:2 and 1:4, following acidification in the presence of citrate, also suggested that the BSP and pancreas activities may be different. That is, whereas the active pancreas fraction precipitated largely between 50 and 67% ethanol, the majority of the BSP antibacterial fraction remained soluble at or above 80% ethanol. In this experiment the BSP fractions were not subjected to heat.

An additional experiment, comparing the ethanol precipitation of an acid- citrate- heat treated BSP-CP eluate (neutralized) with a standard

ethanol precipitation of a pancreas-CP eluate, may have provided additional evidence as to the differences or similarities of the two preparations.

"Purified" pancreas preparations were often composed of two major components, as determined by SDS gel electrophoresis. However, these components had about the same mobility as the component of BSP preparations. The higher citrate concentrations of the pancreas may have altered the mobilities slightly.

The BSP preparations induced a better antibody response than the pancreas preparations. However, one difference between the two antigenic preparations may be of importance. That is, whereas the pancreas preparations were dialyzed before inoculation into rabbits, the BSP preparations were not.

One component (a weak antigen) of the BSP preparation shared identity with a component of the pancreas preparation. No conclusion as to the identity of this component can be made i.e. contaminating protein or active protein.

Although inconclusive, the results of the preceeding experiments, coupled with comparisons of standard ethanol precipitations, suggest that the antibacterial substances of BSP and pancreas are different. However, the similar physico-chemical properties of the two preparations tend to suggest that the two, if different, are closely related proteins of the same family; one may simply be a modified form of the other.

4. Conclusions

Future studies should include further purification of the BSP and pancreas preparations. The BSP antisera could be of value as a reagent for monitoring the purity of a given preparation. When sufficient quantities of homogeneous material are available, amino acid analyses

of seminal plasma and pancreas fractions would be of value. In conjunction, the determination of those features of the molecular structure, which are essential for the antibacterial activity, might lead to the synthesis of a simpler molecule of equal or greater effectiveness which could be used in the treatment and prevention of infection. Such a proposal would certainly necessitate toxicity studies in mammalian tissues.

At the conclusion of this project, the complete nature of the antibacterial factors present in bovine seminal plasma and pancreas remains uncertain. Some comparisons can be made, however, between these and other basic antimicrobial substances reported in the literature. For example, a comparison of the physico-chemical properties of the antibacterial fractions of BSP and pancreas, with those of other basic peptides isolated from mammalian tissues and secretions, suggests that the substances isolated in this study are different from the nondialyzable, cationic proteins isolated from bovine teat canal keratin and milk cells (Hibbitt and Cole, 1968; Hibbitt et al., 1969; Hibbitt et al., 1971). Nor do the two fractions correspond to the nondialyzable, basic proteins from bovine cervical mucus (Brownlie and Hibbitt, 1972); or to the trypsin-insensitive prostatic antibacterial factor isolated by Stamey et al. (1968).

Howard et al. (1975) described a heat stable, dialyzable, mycoplasmacidal compound in bovine whey. However, as discussed previously, incomplete characterization of this substance makes the comparison with BSP and pancreas factors difficult.

The studies of Schollum et al. (1977), involving the ultrafiltration of whole bovine seminal plasma, suggested that the antimicrobial activity in BSP is associated with both large and small components, and that the small forms reaggregate spontaneously to form complexes with molecular weights greater than 50,000 daltons.

Shannon et al. (1974, 1975) suggested that the antibacterial fractions

which they isolated from bovine seminal plasma and other bovine tissues also exist as aggregates. These aggregates, composed of a small monomeric peptide with a molecular weight of approximately 3000 daltons, dissociate under acidic conditions to become dialyzable at pH 3.0.

The serological studies included in this work, clearly demonstrated that the most active BSP preparations, preparations which gave a single band on SDS gel electrophoresis consistent with a molecular weight of approximately 15,000 daltons, did, in fact, contain three major components - all presumably cationic and all of a similar molecular size; there was, however, no evidence for the presence of a small monomeric peptide. Until further studies are completed, the BSP and pancreas factors examined in this work must be considered different from those studied by Shannon et al. (1974, 1975).

In many respects the antibacterial factor of bovine seminal plasma is histone-like, but as shown previously, the amino acid compositions of the BSP factor and various calf thymus histones are not compatable.

Although incomplete, this study provides further evidence that there exists in animal tissues and secretions, a group of basic peptides of similar size and charge which possess antimicrobial properties. Although similar, differences are evident. In many cases these differences may simply represent modifications (perhaps caused by the isolation procedure itself) of a single, cationic, antimicrobial substance, important in the natural defence of an animal host against invading microorganisms.

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