SERUM AMYLOID A AND THE ACUTE PHASE RESPONSE IN BOVINE PNEUMONIC PASTEURELLOSIS

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ABSTRACT

The acute phase response is a cascade of events which occurs immediately after an injury or infection. It is a non-specific, non-immune defence mechanism which consists of a local and a systemic response that vary from species to species. The aim of the work described here was to increase our understanding of the systemic acute phase response (APR) in cattle.

An established experimental model for bovine pneumonic pasteurellosis in which calves were inoculated intra-tracheally with *P. haemolytica* A1 or with lipopolysaccharide (LPS) extracted from the organism was used in these studies of the APR. Serum amyloid A (SAA), a major acute phase protein in cattle was monitored in an indirect enzyme-linked immunosorbent assay developed using a commercially available rabbit anti-human SAA antibody. The assay was standardized using a pool of acute phase bovine serum which was quantified with purified bovine SAA (b-SAA) prepared by hydrophobic interaction chromatography and gel filtration. This allowed b-SAA concentrations in serum to be estimated in SI units with the detection limit of the assay being $3\mu g\ ml^{-1}$.

The APR induced with *P. haemolytica* was characterised within 24 hours by the clinical signs of fever, anorexia, tachypnoea and a dull demeanour and was associated with a marked neutrophilic leukocytosis, hypoferraemia and hypozincaemia. During the same period the endocrine system was affected with an elevation of cortisol and a reduction of thyroxine being detected. The acute phase protein response was manifested by an increase in both SAA and haptoglobin (Hp) concentrations within 24 hours of inoculation and the concentration of both proteins reached a peak at 48 hours post inoculation. However, in studies comparing the effect of different isolates of *P. haemolytica*, on the acute phase protein response only the SAA levels correlated with the pathogenicity of the infectious organism. Frequent sampling during the initial phase of the APR indicated an earlier rise in SAA compared to Hp. In addition, a rise in fibrinogen was found at 24 hours, but there were no clear increases in ceruloplasmin or copper concentrations. Most results from a comprehensive biochemical analysis of sera from infected animals were within the normal range, but plasma concentration of bilirubin was increased and glutamate dehydrogenase activity was decreased.

Animals treated with *P. haemolytica* LPS intra-venously demonstrated an APR which was comparable to that observed when the whole organism was challenged intra-tracheally. In studies to determine the role of bovine cytokines in the infection, both *P. haemolytica* and LPS elicited a tumour necrosis factor (TNF_α) response which peaked 2 hours after challenge and returned to non-detectable levels after a further 4 hours. Unsuccessful attempts were made to measure the plasma concentration of Interleukin 1 and Interleukin 6.

It is concluded that the APR in bovine pneumonic pasteurellosis is characterised by clinical changes and a wide array of pathophysiological alterations which include leukocytosis, mineral redistribution, endocrine and metabolic changes, and an acute phase protein response. The APR varied with the isolate of *P. haemolytica* and appears to correlate with the pathogenicity of the organism. Furthermore, the marked response in TNF_α concentrations implicates this cytokine as a major mediator of the APR in cattle.

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AUTHOR'S DECLARATION

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DEDICATION

This thesis is dedicated to my dearest mother for her devotion, encouragement and moral support without which I could never have undertaken this work.

CHAPTER I

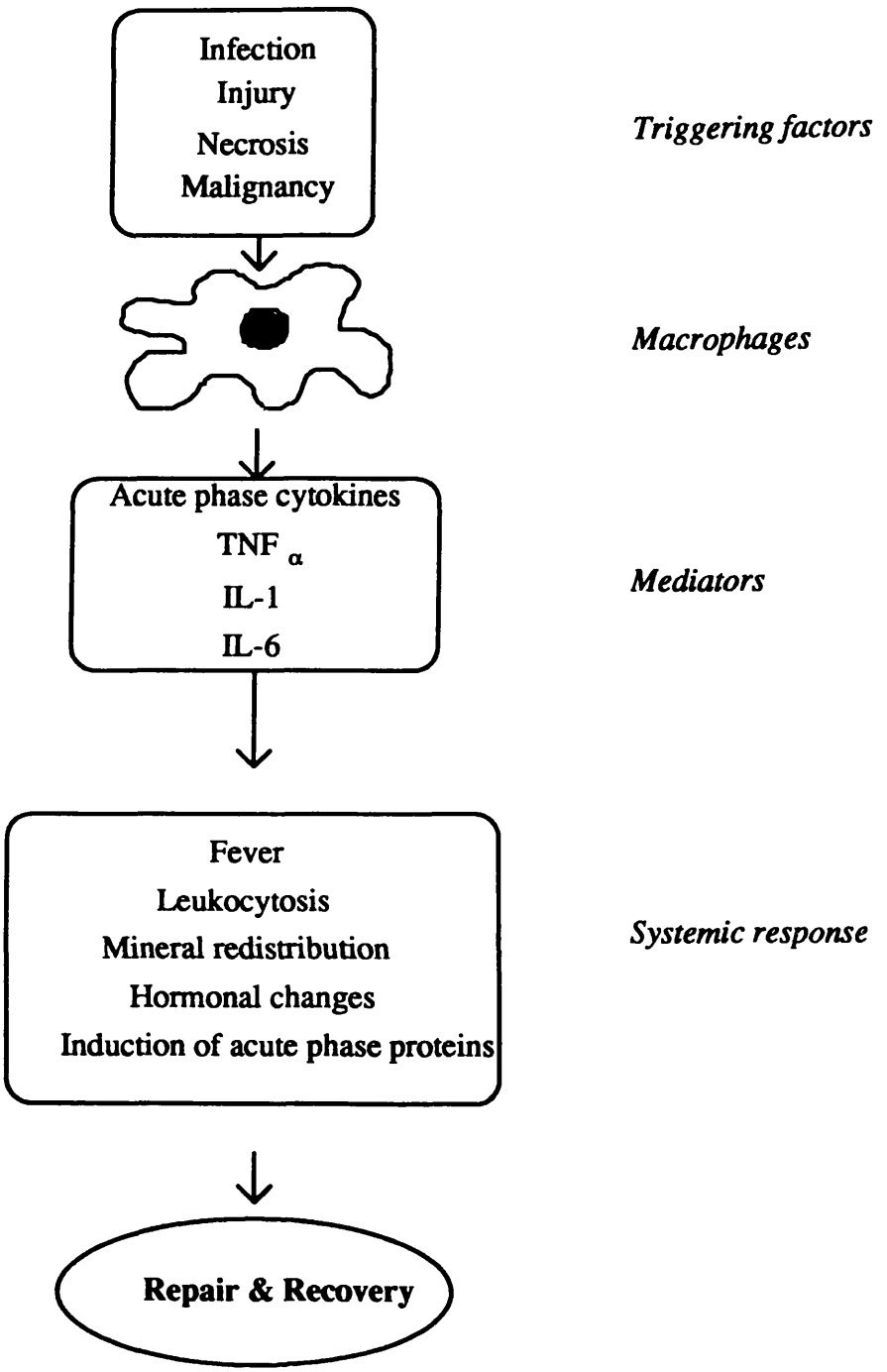
INTRODUCTION

1.1 General introduction

The acute phase response (APR) is a cascade of events initiated by the host as an immediate response to infection, injury or neoplasia. It is a non-specific and a non-immune response which can be divided into two phases; the local response and the systemic response (Gordon and Koj 1985; Whicher and Evans 1992). The local acute phase or inflammatory response which occurs at the site of infection is characterised by dilation of blood vessels, release of lysosomal enzymes and infiltration of inflammatory cells which then produce mediators to induce the systemic APR. This phase encompasses pain, fever, endocrine changes, mineral redistribution and alterations in serum protein concentrations characterised by modified production of liver-derived acute phase proteins (APP) such as fibrinogen, haptoglobin and serum amyloid A (Fig.1.1) (Kushner 1982; Myers *et al.* 1984; Besadovsky *et al.* 1986; Atkins 1989; Pepys 1989; Sipe 1990). The APR also includes systemic metabolic changes such as increased protein catabolism, increased gluconeogenesis and a negative nitrogen balance that facilitate or modulate the local processes of inflammation, for instance by providing energy and substrates for production of leukocytes. These changes enhance the killing of invading organisms, digestion of cell debris by phagocytosis and promote the proliferation of connective tissue cells and repair of the inter-cellular matrix. Taken together, the changes which occur in the systemic response help to protect the host in infection and injury (Sipe 1985b; Stadnyk and Gauldie 1991; Whicher and Westacott 1992).

The APR is essentially a beneficial reaction of the body to tissue injury. It differs from the more delayed immune response by being non-specific and non-immune but like the immune response it is a highly complex process. The primary aim of the APR is to protect the host by restricting damage at the site of injury and restoring homeostasis. Most changes associated with an

Fig.1.1 Acute phase response.



APR occur within hours or at least within days of the tissue damage but these alterations can also be present during persistent or chronic diseases (Gordon and Koj 1985).

The APR in man has been known since antiquity. The ancient Greeks recognised that the red blood cells sedimented faster in peripheral blood from an extremely ill person than that from a normal individual. The increase in erythrocyte sedimentation rate was later revealed to be due to an elevation of fibrinogen and other APP in plasma.

The modern history of the plasma protein acute phase response to tissue injury began in the 1930s with the discovery of C-reactive protein (CRP) by William S. Tillett and Thomas Francis who identified an active compound while working on pneumococcal pneumonia (Tillett and Francis 1930). They found that in the serological responses to various pneumococci extracts one particular extract, which they named C-polysaccharide reacted with sera of acutely ill patients. It was also noted that after the resolution of the clinical crisis the capacity of the patient's sera to precipitate C-polysaccharide rapidly disappeared. In later studies the C-polysaccharide reactive compound was identified as a serum protein and became known as CRP (Pepys 1981). Since the discovery of CRP, numerous studies had been conducted to investigate the role of acute phase proteins in the inflammatory response in both man and in animals (Koj 1974; Pepys 1981; Kushner 1982; Pepys and Baltz 1983; Eckersall and Conner 1988; Whicher and Evans 1992).

The term "acute phase response" was first introduced by Abernethy and Avery (1941) to describe the properties of sera from patients with febrile infectious diseases which contained the C-reactive compound. In 1947, Chanutin and Ludewig described a rise in the concentration of fibrinogen in rats following intramuscular injection of sterile pus demonstrating that fibrinogen was also an APP. However, it was not until the post-war period that

immunological and electrophoretic methods were used to quantitate changes in the concentration of plasma proteins produced during injury or infections (Darcy 1964). In these studies, Darcy (1970) demonstrated by electrophoresis of serum, that following injury, rats produced extra proteins in the alpha-1-globulin region. This and further studies in other species showed that the plasma proteins which increased during infection or injury vary between species (Kushner and Mackiewicz 1987; Fey and Gauldie 1988). Plasma proteins such as CRP in human and α_2 macroglobulin in rats were later defined as "acute-phase reactants" or "acute phase proteins" (Koj 1974; Kushner 1982). The use of the term APR has now become applicable to include several metabolic changes during the post-inflammatory reaction.

In early the 1960, Atkins *et al.* (1964) studied the mediators of the APR. They showed that leukocytes produce protein mediators which they named as 'endogenous pyrogen' (EP). Kampschmidt and Upchurch (1974), by injecting the endogenous pyrogen intramuscularly into rats demonstrated that the animals developed an acute phase response characterised by rise in body temperature accompanied by a depression of iron and zinc. This was the start of the investigations which have led to the conclusion that the acute phase response is mediated by cytokines, notably interleukin 1, interleukin 6 and tumour necrosis factor (Kampschmidt and Upchurch 1974; Kampschmidt and Pulliam 1978).

This introduction is divided into four sections. The first section deals with the acute phase response in general, concentrating on the main features of the systemic response as relevant to this thesis. This section is based on investigations using laboratory animals and studies in man. The second section deals with the APR in domestic animals with particular emphasis on serum amyloid A as a major acute phase protein in cattle. In the third section bovine pasteurellosis is briefly reviewed as this is an important disease of cattle in which a major acute phase response has been observed and was the experimental model used in this study. The final section describes the aims of the study.

1.2 The acute phase response

1.2.1 Local response

The local acute phase response occurs at the site of injury or infection and commences immediately after the insult with an increase in the permeability of the post-capillary venules. This increase in permeability is due to the action of a wide variety of substances such as prostaglandins (PGE_2, PGD_2 and PGI_2) leukotrienes and other vasoactive, kinin-like mediators (Emau *et al.* 1984; Sipe 1985a; Whicher and Westacott 1992). The haemodynamic changes that occur as a result of vascular leakage include platelet aggregation and the early influx of neutrophils, macrophages and other cell types to the site of tissue damage. The vascular leakage also leads to tissue oedema and pain. The cells that arrive at the site of tissue injury and those that are resident, release proteinases and lysosomal enzymes which causes cell necrosis. Many of these changes are mediated by cytokines produced by mononuclear phagocytes. Mononuclear phagocytes are cells derived from the monocytic linearage and include monocytes, Kupffer's cells and peritoneal, pulmonary and other macrophages. Activation of these cells (monocytes and macrophages) are one of the earliest cellular responses to tissue injury and is associated with the release of a variety of inflammatory mediators including arachidonic acid metabolites, complement split products, lysosomal enzymes, oxygen free radicals and cytokines.

1.2.2. Systemic response

This section describes the systemic acute phase response which encompasses a wide range of pathophysiological alterations such as fever, leukocytosis, trace mineral redistribution, increased release of stress hormones and synthesis of hepatic APP.

Studies in man and laboratory animals have demonstrated that cytokines namely IL-1, IL-6, and TNF_α produced by monocytes and macrophages are

responsible for mounting the systemic acute phase response and have been the subject of extensive reviews (Sehgal *et al.* 1989; Kishimoto 1990; Moldawer 1992).

1.2.2.1 Fever

Fever is one of the characteristic features of the systemic acute phase response. In fever, the thermoregulatory 'set-point' is elevated and as a result heat production is increased and concomitantly heat loss is decreased (Snell and Atkins 1967). The first link between systemic fever and an inflammatory lesion was discovered when it was found that fever could be induced by injecting a protein known as 'exogenous pyrogen' isolated from sterile pus (Atkins and Bodel 1972). According to this study, in the host the exogenous pyrogen induces leukocytes to synthesise and release an EP which produced fever. Later studies by Dinarello (1984a) demonstrated that the substance originally identified as EP was interleukin-1.

Moderate fever plays an important role in improving the host's defence against infection as it helps to increase mobility of leukocytes to the site of infection and enhances their phagocyte and bactericidal activities. In addition, the inflammatory and immune responses are known to function optimally under a slightly raised temperature rather than at normal body temperatures (Kluger *et al.* 1975).

1.2.2.2 Leukocytosis

Leukocytosis is another component of the APR which commences within a few minutes of initiation of the inflammatory process (Sipe 1985a). Under normal circumstances, leukocytes in circulation vary from 400 to 11,000 cells per cubic millimeter but during the APR this value can increase by 5- to 10-fold. Neutrophils predominate in the leukocytosis associated with the APR, except in

specific allergies or parasitic infections where eosinophil counts tend to increase (Sipe 1985b).

At the site of infection several pathological changes take place. These include cell necrosis, repair of injured tissues, the removal of waste material and debris and the elimination of invading bacteria. Damaged cells release active products such as lysosomal enzymes, oxygen radicals, prostaglandins and histamine which are toxic to the host (Emau *et al.*. 1984; 1986; Breider *et al.* 1990). The host, in order to protect itself from these toxic compounds, increases blood flow to the site of injury by dilation of blood vessels. The changes in the blood flow allow large number of leukocytes, particularly neutrophils to be brought to the site of injury (Walker *et al.* 1985; Bowersock *et al.* 1990). These neutrophils, which have an antimicrobial action make their way between endothelial cells to arrive at the site of tissue injury where they initiate destruction of invading bacteria by the generation of oxygen free radicals and lysosomal enzymes (Strieter *et al.*. 1989; Breider *et al.* 1991; Whiteley *et al.* 1992). Macrophages follow later as they move much more slowly than the neutrophils, but both these cells when activated, phagocytose bacteria and cell debris (Strieter *et al.* 1992).

During the APR, endotoxins, IL-1, neutrophil releasing factor, prostaglandins and several other complement factors activate the bone marrow to release polymorphonuclear leukocytes and stimulate the maturity of these cells resulting in an elevation of both mature and immature neutrophils in circulation (Sipe 1985a; Whicher and Westacott 1992; Maheswaran *et al.* 1993). The neutrophils migrate rapidly to the site of tissue damage, as described above, and therefore, the rise in circulating neutrophils is transitory since their initial increase is followed by a sharp fall as the rate of replacement fails to cope with the rate of migration from the circulation.

1.2.2.3. Trace minerals

Another component of the non-specific host defence response to infection involves changes in serum trace mineral concentrations. Specific alterations include an elevation of plasma copper and depression of both zinc and iron concentrations. Many of these alterations in the storage and binding of trace metals have been attributed to the activity of IL-1 (Kampschmidt and Upchurch 1969; Kampschmidt and Pulliam 1978). The redistribution of trace elements in combination with an elevation in body temperature appears to play a major role in containing bacterial proliferation (Kluger and Rothenburg 1979).

Iron

Apart from the iron present in the porphyrin ring of haemoglobin the major portion of iron in the circulation is bound to transferrin (Bothwell *et al.* 1979). In the APR, the early increase in microvascular permeability allows albumin and plasma proteins of similar size, such as transferrin to rapidly leak out of the circulation resulting in reduced serum concentrations (Whicher and Westacott 1992). This reduction of transferrin concentration is believed to contribute significantly to the decrease in serum iron concentration during the APR (Fleck and Myers, 1985). In man, a significant fall in plasma iron and transferrin concentrations were observed 6 hours following elective surgery, and continued to be reduced to 80% of the normal level at the end of 12 hours (Kampschmidt and Upchurch 1969). However, in a subsequent study by the same group (Kampschmidt and Upchurch 1974) it was shown that in elective surgery, the fall in serum iron concentration began even before a drop in transferrin commenced (Kampschmidt and Pulliam 1978). According to the latter study, this reduction in iron concentration is due to an increase in uptake by the liver which may be related to IL-1 activity (Kampschmidt 1984; Fleck 1985). According to Bennett and Kokocinski (1979) a further reason for the fall in serum iron during injury may be due to the action of lactoferrin, an iron binding protein released from neutrophils which forms a complex with the iron released from transferrin and is then taken up

by the liver. van Snick *et al.* (1974) reported that the transfer of iron from transferrin to lactoferrin occurs only in an acidic environment therefore, it is most likely that this transfer occurs at the site of injury where an acidic pH prevails. Iron is essential for bacterial growth and increases *in vitro* iron, and zinc deprivation has proved to be inhibitory for most pathogenic bacteria (Fleck and Myers 1985). The redistribution of the iron in the APR increases competition between the host and the microbe for this trace element which could eventually limit bacterial proliferation (Kluger and Rothenburgh 1979). In addition, the rapid sequestration of iron by the action of lactoferrin at the inflammatory site may also serve as an antimicrobial mechanism to curtail multiplication of the bacteria during an infection (Bullen 1981).

Zinc

In man, 65% of plasma zinc is bound to albumin while the balance is bound to α_2-macroglobulin (Giroux 1975). Following acute trauma, the loss of albumin from the vascular compartment is accompanied with reduction (approximately 40%) of zinc, particularly during the first 8 hours (Kampschmidt and Pulliam 1978). Serum zinc could also be reduced due to the action of IL-6 activation of hepatocytes during the APR to synthesise metallothionine, the Zn storage protein in liver (Fleck and Myers 1985). Normally, metallothionine synthesis is dependent on the intake of dietary zinc but in the APR glucocorticoids, catecholamines, glucagon and IL-6 induce metallothionine production there by increasing the hepatic uptake of zinc from the circulation (Schroeder and Cousins 1990). In man, the rapidity of response of trace metals to infection was demonstrated by Pekarek *et al.* (1972) where it was shown that the serum zinc levels fell even before the clinical symptoms of viral infections became apparent. As in the case of iron, zinc is an important requirement for the microbial growth thus low plasma zinc concentrations benefits the host by limiting microbial proliferation.

Copper

In contrast to serum zinc and iron, the copper concentration in circulation is elevated in the APR due to an increased synthesis of ceruloplasmin, the copper binding protein (Fleck and Myres 1985; Auer *et al.* 1989). The presence of elevated copper concentrations in acute and chronic infectious conditions in human patients has been known for many decades but the specific role of copper in the APR is yet not clear (Fleck and Myres 1985).

1.2.2.4. Hormones

Alteration of hormone concentration manifested by an increase of ACTH, glucocorticoids, glucogen, catecholamines and a reduction of thyroid hormones is another major feature of the APR (Stadnyk and Gauldie 1991; Whicher and Westacott 1992; Wong and Hershman 1992). During bacterial infection, cytokines stimulated by endotoxin promote interaction between the neurological and endocrine pathways through the activation of the hypothalamus. This results in the release of corticotrophin releasing factor which in turn activates the anterior pituitary to release ACTH.

Extrapituitary pathways are also known to stimulate corticosteroids (Whicher and Westacott 1992). Endotoxins can directly stimulate the adrenal cortex to release corticosteroids (Whitcomb *et al.*. 1988). Salas *et al.* (1990a) reported that the cytokines IL-1 and IL-6 are also capable of stimulating the release of cortisol directly from the adrenal cortex. In experimental studies, Besedovsky (1986) showed that recombinant human IL-1β by itself can increase ACTH and cortisol concentration in mice. Furthermore, *in vitro* studies have shown that corticotrophic cells from the pituitary when incubated with IL-1 release ACTH (Bernton *et al.* 1987). In the APR the anti-endotoxin and anti-shock functions of glucocorticoids assist to overcome the harmful effects of LPS (Finley and McKee 1982).

The reduction of thyroid hormones in the acute phase response is thought to be related to the lethargy associated with the APR. Low thyroxine (T_4) levels have been reported in man during acute infections although the specific role is yet unclear (Chopra *et al.*. 1979; Kaplan *et al.* 1982; Semple 1986; Wong and Hershman 1992). Recent studies in man have identified the presence of large numbers of TNF_α receptors in the thyroid gland during the APR indicating that the fall in T_4 may be associated with the release of TNF_α at this time (Wong and Hershman 1992).

1.2.2.5. Acute phase proteins

Acute phase proteins are a group of plasma proteins whose concentration increases by 25% or more in the first 7 days following tissue damage. These are liver derived glycoproteins (Kushner 1982; ICSH Expert Panel 1988; Evans and Whicher 1992). As in other systemic responses the cytokines IL-1, IL-6 and TNF α are the prime mediators in stimulation of hepatic acute phase protein synthesis. It is possible that each cytokine induces a different set of APP (Thomson *et al.* 1992). The acute phase proteins of man and laboratory animals have been extensively reviewed (Kushner 1982; Koj 1985; Gauldie *et al.*. 1987; Sipe 1992) and some of the more studied APP are shown in Table 1.1. which also gives their function, where this is known.

Acute phase proteins serve various roles in inflammation including mediators, modulators, inhibitors, scavengers and, immuno-modulators. APP are designed to contribute to the repair and restoration of the damaged tissues. In certain types of diseases specific APP may be more actively catabolized than other types leading to low plasma concentrations. For example in a haemolytic crisis haptoglobin binds to haemoglobin forming a haptoglobin-haemoglobin complex which is then absorbed by the liver reducing the plasma concentration of haptoglobin.

Table 1.1 Important functions of some acute phase proteins

Protein	Function	References
α_1 Acid glycoprotein	Transport	Koj (1985)
Ceruloplasmin	Scavenger of superoxide radicals, prevent autoxidation of lipid	Goldstein *et al.* 1982
C-Reactive Protein	Opsonin	Pangburn and Muller-Eberhard 1984
Fibrinogen	Participates in coagulation and wound healing	Pangburn and Mueller-Eberhard 1984
Haptoglobin	Scavenger of haemoglobin	Allison and Reed 1957
α_2-Macroglobulin	Antiproteinase	Lonberg Holm *et al.* 1987
Serum Amyloid A	Precise function unknown; participates in reverse cholesterol transport?	Benditt *et al.* 1982

Models of inflammation in laboratory animals have been used extensively to study the APR of the rat, rabbit and mouse (Gordon and Koj 1985). Each species exhibits a unique pattern of APR in relation to APP synthesis Table 1.2. (Kushner and Mackiewicz 1987). In rabbits both CRP and SAA are inducible APP while in mice, only SAA increases on stimulation with no significant alteration in CRP (Pepys and Baltz 1983; Sipe 1990). Conversely, serum amyloid P (SAP), another protein defined by its association with amyloid deposits, is a major APP in mice, but not in rabbits or man. The acute phase protein profile of the rat differs markedly from other laboratory animals and from that reported in man (Kushner and Mackiewicz 1987; Sipe 1992). A major APP in rat serum was characterised by Darcy (1964) which was first known as "Darcy Protein", and later renamed as "cysteine proteinase inhibitor" based on its function. α_2-Macroglobulin, a generalized proteinase inhibitor is another major APP in the rat (Fey and Gaulgie 1988) while both SAP and CRP are moderate APP in this species (Pepys and Baltz 1983). In the rat the primary structure of SAA protein is different from other species of laboratory animals and is uniquely resistant to inflammation associated amyloidosis (Baltz *et al.*. 1987). In some species, plasma concentrations of albumin and transferrin have been shown to fall during the acute phase response and are known as negative acute phase proteins.

1.2.3. Cytokines

Communication between cells is essential for biological functions. An important mode of cell communication, essential for protection from infectious agents occurs through a group of soluble mediators known as cytokines. These are proteins which are synthesised by a variety of cells including monocytes/macrophages, fibroblasts and endothelial cells in response to specific stimuli such as cell proliferation, cell differentiation and mediators of the APR. Most cytokines function pleiotropically exhibiting a wide range

Table 1.2 Acute Phase Proteins (APP) in different species

Species	Major	Moderate	Minor	No change	Negative	References
Man	CRP; SAA; LBP	Hp; α_1AGP; Fb		SAP α_2-Mac	Alb; Tf	Kushner 1982 Tobias *et al.* 1986
Cattle	Hp; SAA	Cp; α_1AGP; α_2-Mac Fb; CRP; LBP			Alb; Tf	Eckersall 1992a
Sheep	Hp; SAA?	Cp; Fb; α_1AGP				Pfeffer and Rogers 1989
Horse	CRP; SAA	Hp; Fb; α_1AGP				Pepys *et al.* 1989
Pig	Hp; CRP					Eurell *et al.* 1990 Burger *et al.* 1992
Dog	CRP	α_1AGP				Conner *et al.* 1988a
Cat		CRP?				Watanabe *et al.* 1992
Rabbit	CRP; SAA; LBP	α_2-Mac	Tf			Gordon and Koj 1985 Tobias *et al.* 1986
Rat	α_2-Mac	α_1AGP; SAP	CRP	SAP	Tf (-/+)	Kushner 1982
Mouse	SAA;	SAP; α_1AGP	CRP LBP		Tf	Gordon and Koj 1985 Gallay *et al.* 1993

α_1AGP-α_1Acid glycoprotein; Alb-Albumin; CRP-C-Reactive Protein; Fb-Fibrinogen; Hp-Haptoglobin; LBP-Lipopolysaccharide binding protein; α_2-Mac α_2-Macroglobulin; SAA-Serum amyloid A; SAP-Serum Amyloid-P protein; Tf- Transferrin

of biological effects on cells (Table 1.3) while, on the other hand, a particular cell can also be influenced by different cytokines. Cytokines have a very short half-life ensuring that they act only for a limited period in circulation or in extracellular fluid (Kunkel and Remic 1992). The classification of cytokines is based on their physiological role; those that activate cell growth are referred to as growth factors while those that are produced by or act upon cells of the immune system are known as lymphokines, and cytokines which are implicated in tumour destruction are termed tumour necrosis factors. Interleukins are a particular group of cytokines produced and released by activated T lymphocytes that act on other lymphocytes to produce biological effects (Clemens 1991).

It is possible for each cytokine to induce a different aspect of the systemic APR (Evans and Whicher 1992; Thompson *et al.*. 1992). For example TNF_{α} activates the early APR. IL-1 plays a major role in inducing fever, whereas IL-6 has a greater effect on hepatocytes to stimulate APP synthesis. Many biological actions are the result of synergistic action of two or more cytokines (Atkins *et al.*. 1964; Atkins and Bodel 1972; Le and Vilcek 1989).

Previous studies in man and laboratory animals have demonstrated that specific cytokines are invovled in the regulation of the APR and there are several recent reviews on this topic (Le and Vilcek 1987; Sipe 1990; Moldawer 1992). IL-1, IL-6 and TNF_{α} are the major cytokines which are responsible for mounting an APR following tissue injury and infection. For example in Gram-negative sepsis, bacteria invade the host and multiply in the circulation, resulting in a rise in the serum endotoxin concentration. This endotoxin induces the synthesis of TNF_{α} in monocytes which is released into the circulation 1-2 hour later. IL-6 is released in a similar manner 30-60 minutes after the TNF_{α} response partly caused by TNF_{α} and IL-1 stimulation of monocytes and

Table 1.3 Biological responses and tissue sources of TNF_{α}, IL-1 and IL-6

	TNF_{α}	$IL\text{-}1_{\alpha}$ and $_{\beta}$	IL-6
Fever	Yes	Yes	Yes
Leukocytosis	Yes	Yes	Yes
Induction of acute phase response	Yes (weak compared to IL-1 and IL-6	Yes	Yes (very strong)
Anorexia/weight loss	Yes	Yes	Weak
Skeletal muscle proteolysis	Yes	Yes	Unknown
Induction of cytokine synthesis	Yes for IL-1 and IL-6	Yes for IL-6 Weak for TNF_{α}	Suppresses TNF_{α}
ACTH synthesis/activation of adrenal cortex	Yes	Yes	Yes
Tissue source	M, Mac, E, F, K	M, Mac, E, F, K	M, Mac, F, K

M-monocytes; Mac-macrophages; E-endothelial cells; F-fibroblasts; K-kupffer cells

endothelial cells. The release of IL-1 in serum probably occurs between those of TNF_{α} and IL-6 (Waage *et al.* 1989; Waage *et al.* 1992). The interaction of these cytokines is believed to be important in the stimulation of the systemic APR (Hesse *et al.* 1988; Michie *et al.* 1988; Shalaby *et al.* 1989; Rothe *et al.* 1993).

1.3. Acute phase response in domestic animals

1.3.1. Acute phase protein response

The APP response varies with the species (Table 1.2; Kushner1982; Conner *et al.* 1988a; 1988b; Eckersall 1992a). In cattle, Hp and SAA are recognised as major APP (Eckersall and Conner, 1988; Boosman *et al.* 1989). Although fibrinogen has been widely used in veterinary clinical diagnostic work, it only shows a medium response in cattle (Eckersall and Conner 1988; Kent 1992) (Table 1.2). The use of acute phase proteins for veterinary diagnosis has been recently reviewed by several workers (Eckersall 1992a; Kent 1992; and Gruys *et al.*. 1993). A further potentially important use of APP is in ante-mortem examination to identify infected carcases and thereby to improve the quality of meat for human consumption (Saini and Webert 1991; Eckersall 1992b).

1.3.1.1. Horse

In the horse, early investigations on the APR were focused on studies relating to the APP response. Serum Hp was one of the first acute phase proteins to be measured in order to detect a variety of clinical conditions in horses (McGuire *et al.*. 1969). In these studies a reduction in Hp concentration was found to be a sensitive marker to detect the presence of intravascular or extravascular haemolysis during virus-induced equine infectious anaemia. In another study, Willett and Blackmore (1979) attempted to use Hp measurements to assess liver function in horses but they failed to demonstrate a definite

correlation between hepatocellular damage and Hp concentration. The use of serum Hp as a clinical test was further studied by Sheldrick *et al.*. (1982) and Kent and Goodall (1991). These workers noted that increased Hp concentrations were more sensitive than leukocyte counts in detecting tissue damage, particularly when associated with bacterial infections. Hp levels were also found to decrease in response to treatment with antibiotic therapy. Sheldrick *et al.*. (1982) concluded that, in the absence of a haemolytic crisis, Hp could be used as a positive acute phase indicator to monitor the health status of horses.

In the studies conducted by Pepys *et al.*. (1989) SAA was recognised as a major APP in the horse and it was found to be a more reliable disease marker compared to Hp to detect viral and bacterial infections. They also noted that SAA concentrations could be used to monitor the general health status of horses in training and racing.

Prealbumin protein (Pr) and ceruloplasmin are two other acute phase proteins which have been reported to be sensitive disease markers in the horse (Ek 1980; Barton and Embury 1987). In a clincal study, Ek (1980) measured the Pr by an immunodiffsion technique and demonstrated a significant increase of Pr in horses with acute infections and malignant tumours. Barton and Embury (1987) in an experimental study on the pathogenesis of *Rhodococcus equi* infection in foals showed that the elevation of both serum copper and ceruloplasmin oxidase levels to be sensitive markers of infection.

More recent studies on the APR in horses have concentrated on the cytokine response to experimentally induced endotoxaemia (Mackay and Lester 1992; Hawkins *et al.*. 1993; Carrick *et al.*. 1993). In these investigations injections of LPS or recombinant cytokine preparations have been administered directly into the circulation or to target organs such as joints. The consequent APR has been evaluated by measuring fever, leukocytosis and analysing the cytokine responses. Hawkins *et al.*. (1993) injected LPS into the synovial cavity to measure the APR in the horse. Their studies revealed that IL-6; TNF_{α} and

prostaglandin E_2 were released in response to the subsequent synovitis and that the serum TNF_α concentration peaked at 2 hours and the IL-6 peaked later at 8 hours post-injection. PGE_2 responded immediately and was significantly high for 66 hours post injection. Typical acute phase reactions such as increased rectal temperature, leukocytosis and plasma protein changes were noted. Their study reported that TNF_α is the key mediator of APR in the horse. In another study Carrick *et al.*. (1993) induced endotoxaemia by injecting LPS from *E. Coli* to 2 groups while a third group was injected with platelet activating factor (PAF) receptor antagonist prior to LPS treatment. They monitored the consequent APR activity by measuring the temperature, leukocyte counts, heart rate, packed cell volume, capillary refill time, mean arterial pressure and serum lactate concentrations. The APR developed in all horses in all 3 groups but the group that received PAF receptor antagonist demonstrated a mild APR.

1.3.1.2. Pig

Experimental studies of acute respiratory diseases and atropic rhinitis caused by *Pasteurella multocida* have identified Hp as an acute phase protein in pigs (Eurell *et al.*. 1990; Hall *et al.*. 1992). In addition Hp concentration has also been recognised as an indicator of weight gain in pigs (Eurell *et al.*. 1992). Eckersall (1987) in a preliminary clinical study noted an increase in the Hp, seromucoid and CRP concentrations of weaned pigs with pyrexia resulting from a variety of clinical conditions. More recently Burger *et al.* (1992) suggested that CRP is a good marker to monitor the general health status of pigs following a clinical study involving 120 weaned female piglets in which they observed an increased CRP concentration in animals with fever and inflammatory conditions compared to normal piglets.

1.3.1.3. Sheep and Goat

There have been only limited investigations of the APR in sheep. However, Hp, fibrinogen and ceruloplasmin have been identified as acute phase proteins in sheep following experimental bronchial obstruction (Pfeffer & Rogers 1989) and the response profile resembled that of cattle. In a clinical study, the concentration of Hp was elevated in dystocia cases where dead lambs were present *in utero* as opposed to ewes which had live lambs delivered (Scott *et al.*. 1992). It is evident that the APR takes place in sheep and that the determination of the APP could play a role in detecting inflammatory conditions and monitoring progress after medication.

van Miert *et al.*. (1982; 1983; 1984; 1990; 1992) in a series of experiments studied the APR in goats following adminstration of a range of inducers which included *E. coli* endotoxin, *Trypanosoma brucei,* various interferons and preparations of recombinant bovine and human TNF_{α}. The APR observed varied with the stimulant but in general, the changes observed include an increased heart rate, anorexia, leukocytosis, hypoferraemia, hypozincaemia and hypercupraemia. Hypoglycaemia was observed at the onset of APR which was followed by hyperglycaemia at a later stage. These studies however, failed to examine the hormonal changes or the APP responses which are usually manifested in APR.

1.3.1.4. Dog and Cat

Many APP including ceruloplasmin, fibrinogen, haptoglobin are glycoproteins, therefore the APR can be monitored by a non-specific assay of serum glycoproteins. The APR in dogs was identified by an increase in serum glycoproteins by concanvalin A binding following surgical trauma, endotoxin administration and various inflammatory conditions (Eckersall *et al.* 1985). Several individual APP such as CRP and Hp have been identified in the dog (Conner and Eckersall 1988; Solter *et al.* 1991). Conner *et at.* (1988a) studied the

relative and sequential changes of five APP following surgical trauma in the dog. These investigations demonstrated a 100-fold increase of CRP which peaked 24 hours after surgery followed by peak levels of ceruloplasmin, Hp, and seromucoid which were detected between the fourth and sixth day after surgery. There was little variation in the levels of α_1 antitrypsin. Measurement of CRP levels as a clinical test to detect inflammatory conditions in the dog has been restricted as the assay available was based on an immunodiffusion technique which yielded only a semiquantivative estimate of the protein. This limitation was overcome with the development of an immunoturbidimetric assay which could be performed on most modern biochemical analysers (Eckersall *et al.*. 1991). Measurement of serum Hp and ceruloplasmin concentrations have also been found to be useful markers for detecting inflammatory conditions in the dog (Solter *et al.* 1991).

Little is known on the APP response in the cat. Watanabe *et al.*. (1992) isolated CRP from normal cats serum using an affinity chromatography method and made a detailed analysis of the biochemical characteristics of the protein. They also measured the CRP concentration in cat serum using an immunodiffusion method and noted that the CRP value in the normal cat was higher than that reported for man (Clause *et al.* 1975) and the dog (Caspi *et al.* 1987). However, it is not yet known whether CRP is an APP in the cat. (Watanabe *et al.*. 1992).

1.3.1.5. Cattle

Studies on the APR in cattle have concentrated mainly on investigations relating to the APP (Conner *et al.* 1989). Fibrinogen is the most widely known APP in cattle and is used to detect inflammatory conditions although it is considered to be a moderate APP when compared to other APP such as Hp and SAA (Table 1.2). Bremner (1964) was the first to show that Hp which is undetectable in normal serum is increased

markedly in inflammatory conditions and that caution should be taken in the interpretation as the serum levels could fall during a haemolytic crisis because of an increased hepatic uptake of Hp/haemoglobin complexes by the liver. Spooner and Miller (1971) in a subsequent study, investigated the Hp concentration in a large population of clinically normal and diseased animals and suggested that an assay for Hp can be a useful diagnostic test to detect inflammatory disorders in cattle. Makimura and Suzuki (1982) developed a colorimetric assay for bovine Hp based on the ability of Hp to prevent the destruction of peroxidase activity of haemoglobin in an acid medium. In the studies conducted by Conner *et al.* (1988b) the sequential changes of five APP in cattle were examined following inflammatory stimulation by subcutaneous injection of oil of turpentine. These studies indicated raised levels of α_1 antitrypsin, ceruloplasmin, fibrinogen, Hp and seromuciod on the second and third day after injection, with a peak occurring between the fourth and seventh day. They also noted that the levels of Hp and seromucoid varied with the dose whereas no changes were present in ceruloplasmin and α_1 antitrypsin concentrations. In a further experimental study conducted by Conner *et al.* (1989), increased levels of Hp were observed following inoculations with both *Pasteurella haemolytica* serotype A1 and *E.coli* LPS but there was little Hp response to *Ostertagia ostertagi* infection. The use of Hp as clinical test was examined by Skinner *et al.*. (1991) who demonstrated increased concentrations of Hp in animals with bacterial infections but not in healthy animals or those with metabolic diseases such as ketosis and milk fever. Recent modifications to the Hp assay procedure have allowed it to be adopted as an automated assay in biochemical analysers and this has further improved the potential of the Hp assay being used as a routine clinical test to detect inflammatory conditions in cattle (Skinner 1992). For many years the Hp concentration in bovine serum was expressed as a percentage of Hb binding

capacity: however the purification of bovine Hp by Eckersall and Conner (1990) has now allowed the quantitaion of serum Hp in grams per litre.

Studies conducted by Boosman *et al.*. (1989) have demonstrated that bovine serum amyloid A (b-SAA), a precursior of amyloid protein A (AA), increased by a 100-fold in cattle experimentally injected with *E. coli* LPS. These findings (Boosman *et al.*. 1989) indicate that SAA is a major APP in cattle as it is in man (Pepys and Baltz 1983; Eriksen and Benditt 1986) and that it has a potential of being used as a disease marker in cattle. The measurement of b-SAA was performed using an ELISA system in which a bovine anti-AA (b-AA) antibody was used as the primary antibody. In this ELISA system the SAA concentration was measured using arbitrary units. This has restricted comparison of results with other laboratories. Also anti-AA antibody might not be a suitable antibody to measure circulating b-SAA levels. As reported by Niewold *et al.* (1987) there are specific amyloidogenic SAA-isotypes which activate amyloidosis and they are different to those that increase during the APR. Thus anti-AA should be specific for SAA isotypes which are important to induce amyloidosis and use of anti-AA to measure b-SAA in the APR might give false results. The fact that SAA concentrations in serum are not affected by haemolysis makes it more suitable than Hp as a marker for the APR (Eckersall 1992a; Kent 1992).

The b-SAA concentrations in healthy and diseased cattle were measured by both Alsemgeest *et al.* (1992) and Gruys *et al.*. (1993). These studies showed that the SAA concentration varied with the disease state. In bacterial infections and in Gram-negative sepsis SAA concentrations were close to a maximum of 100 arbitrary units while minor surgical conditions showed only a moderate increase.

Other acute phase proteins studied in cattle include ceruloplasmin (Piercy 1979; Conner *et al.*. 1986, 1988b, 1989) α_1-Acid Glycoprotein (Motoi *et al.* 1992) α_1 antitrypsin (Conner *et al.*

1986, 1988b) seromucoid (Conner *et al.* 1986, 1988b), and fetuin (Dziegielewska *et al.* 1992).

1.3.2. Trace minerals

Several studies in domestic animals, particularly in ruminants have demonstrated alterations in trace mineral concentrations in the APR (Corrigal *et al.* 1976; Verheijden *et al.* 1983b). Experimental studies conducted by van Miert *et al.* (1982) in goats in which the APR was induced after intravenous injection of endotoxin showed an 85 per cent reduction of both plasma zinc and iron concentrations. In another study Boosman *et al.* (1989) reported the development of hypozincaemia and hypoferraemia 17 hours after the administration of *E.coli* endotoxin to calves. They also reported that the concentrations of the two trace elements rapidly returned to the normal levels and that a second injection of endotoxin did not alter the plasma zinc level. Studies reported by Piercy (1979) have shown that calves infected with a pathogenic strain of *Salmonella dublin* had developed an acute hypoferraemia 24 to 48 hours after challenge and that it coincided with the onset of the bacteraemia and pyrexia.

In ruminants the fall in plasma iron and zinc levels in the APR is associated with an increase in copper concentration (Corrigall *et al.* 1976). Pekarek *et al.* (1972) reported a similar pattern of trace mineral alteration in man with viral infections. The inverse relationship between zinc and copper in ruminants was demonstrated in a clinical study in cattle and sheep in which it was shown that in acute infections in ram lambs the plasma zinc level dropped to its lowest level before the copper concentration achieved a peak (Corrigall *et al.* 1976). According to Beisel and Sobocinski (1980) the mechanism by which these effects are produced is attributed to the production of a heat labile factor (later identified as IL-1) by phagocytosing cells which when injected into normal animals enhances liver mRNA synthesis resulting in the production of a

variety of glycoproteins, one of which is ceruloplasmin. This sequence of events results in a rise in ceruloplasmin and hence copper.

Although the potential of using the altered concentration of trace mineral concentrations in the APR as disease marker in ruminants was emphasised by Corrigall *et al.* (1976), few studies have been carried out to fully explore this possibility. In the clinical study conducted by Corrigall *et al.* (1976) cattle with acute infections showed raised plasma copper concentrations and low zinc levels while animals with chronic conditions in which there was little or no systemic illness exhibited only a slight change in the copper and zinc values.

1.3.3 Hormones

Species vary greatly in their response to glucocorticoids, with some species (rat, mouse, rabbit) being more sensitive to glucocorticoids than others (human, monkey, guinea pig). Cattle respond as a corticosteroid-resistant species (Roth 1985). Parturition (Hudson *et al.* 1975), endotoxin-induced mastitis (Paape *et al.*. 1974) and neonatal diarrhoea (Roth 1985) are among the stressful conditions associated with increased plasma cortisol concentrations in cattle.

Alterations in metabolic hormones such as cortisol in the APR in cattle have been the subject of few studies (Elsasser 1992). However, studies of bovine pneumonic pasteurellosis (the subject of the next Section) have shown that increased cortisol is associated with this condition in field cases (Roth 1985). Indeed bovine pneumonic pasteurellosis (BPP) is one of the best examples of stress-associated infectious disease in calves due to its association with high cortisol levels (Roth 1985). Various infectious agents have been associated with this disease, also known as shipping fever pneumonia, but for a long time no single agent was consistently found or able to reproduce the syndrome. A factor generally present is stress and a working hypothesis for the pathogenesis of shipping fever, which was first advanced by Hoerlein and Marsh (1957), proposes that stressors encountered by the animal reduced its resistance mechanisms sufficiently to allow viral and secondary bacterial infections to occur. Many of

the stresses associated with a predisposition to shipping-fever pneumonia are known to cause increased plasma cortisol concentrations. Some of these conditions are weaning, handling, acute pain and transport. The high plasma cortisol associated with BPP could lead to immunosuppression. However, increased plasma cortisol is probably not the only mechanism for stress-associated immunosuppression in shipping fever, but it is undoubtedly an important contributory factor.

1.3.4. Bovine Cytokines

The concentration of tumour necrosis factor in calves after separate challenges of *S. typhimurium* and *E coli* LPS has been demonstrated by Peel *et al.* (1990). In this study TNF_{α} levels began to rise as early as 30 minutes after administration of the LPS, reached peak levels between 1-2 hours post-treatment and returned to baseline 4 hours later. In another *in vivo* study Kenison *et al.* (1991) demonstrated similar TNF_{α} responses following the administration of *E-coli* LPS. However, the calves infected with *S. typhimurium* failed to show a TNF_{α} response in spite of proven septicaemia on blood cultures. Recently, two groups of workers reported conflicting results from experiments designed to study the systemic TNF_{α} response in calves infected with *P. haemolytica.* Pace *et al.* (1993), using a bioassay, demonstrated an increase in circulating TNF_{α} following intra-tracheal inoculation of *P. haemolytica.* The rise in the TNF_{α} concentration was observed within 2 hours of challenge and lasted for 48-72 hours. In contrast to these observations Espinasse *et al.* (1993), using a radioimmunoassay, were unable to detect any increases of serum TNF_{α} concentrations in calves inoculated with *P. haemolytica by* intra-nasal/intra-tracheal route as described by Gibbs *et al.* (1984). In the latter study TNF_{α} levels were measured in serum collected at 6 hourly intervals over a period of 3 days after treatment.

There have been few reports on the IL-1 and IL-6 in cattle (Collins *et al.* 1989; Shuster *et al.* 1993; Nakajima *et al.* 1993). Collins *et al.* (1989) reported

IL-1 like activity after using intra-mammary devices. IL-1 like activity has also been reported in milk after experimental udder infection by intra-mammary administration of LPS (Shuster *et al.* 1993). Nakajima (1993) was able to detect an APR in cattle following repeated infusion of human recombinant IL-6.

1.4 Bovine Pasteurellosis

1.4.1. Introduction

Pasteurellae are important bacterial pathogens of domestic animals, in which they cause a variety of diseases including pneumonia, septicaemia and mastitis (Carter 1967). Within the genus Pasteurellae the most important respiratory pathogen of cattle is *P. haemolytica* which is responsible for bovine pneumonic pasteurellosis (BPP), an economically important disease to the farming industry in Western Europe and North America (Dagleish 1989; Frank 1989; Whiteley *et al.* 1992). Currently, BPP costs over one billion dollars per annum to the beef cattle industry in the USA (Whiteley *et al.* 1992) and in the UK, the annual estimated losses due to BPP and other infectious respiratory diseases in cattle is almost £30 million (Dalgleish 1989).

P. haemolytica is a Gram-negative, non-motile facultatively anaerobic bacterium which displays characteristically weak haemolysis on blood agar. The species can be separated into two biotypes, A and T, on the basis of several *in vitro* criteria including carbohydrate fermentation reactions (Smith 1961), nucleic acid homology (Biberstein and Francis 1968) and antibiotic sensitivity (Biberstein and Kirkham 1979). Clinically, the A biotypes are associated with pneumonic pasteurellosis in ruminants and the T biotypes with systemic disease in weaned lambs (Smith 1961). Sixteen serotypes are differentiated by an indirect haemagglutination (IHA) test which depends on the capsular antigens of the bacteria (Biberstein 1978; Fodor *et al.* 1987). The serotype A1 is the most frequently isolated serotype in BPP (Whiteley *et al.* 1992)

Bovine pneumonic pasteurellosis which is also referred to as transit fever, stockyard fever or shipping fever was first described in the United States in 1915 by Kinsley (1915). The causative agent of BPP was then referred to as *Bacillus bovisepticus* and it was suggested that the organism was universally distributed but rarely produced the disease unless the animal's resistance was diminished. Kinsley (1915) also stated that young cattle developed this disease following movement and mixing with different populations. The first description of BPP in the UK was made by Hepburn (1925) when he noticed the disease in cattle 7-10 days after they had arrived in Aberdeen following a journey by ship from Ireland *via* the Orkney islands. On post-mortem examination, Hepburn observed the presence of fibrinous pneumonic lesions in the apical lobes and recovered the bipolar organism which had been described previously by Kinsley (1915). Many years later the bacterium isolated from animals with transit fever was renamed as *Pasteurella haemolytica* by Carter (1956).

In Britain, BPP is primarily a disease of recently weaned, newly housed, single-suckled beef cattle (Selman 1986; Dalgleish 1989). Several factors are known to predispose calves to the disease and these include extremes of temperature, housing in poorly ventilated buildings, sudden changes of feed, primary respiratory virus infections (Parainfluenza 3, Infectious bovine rhinotracheitis) and stress induced by transport and mixing of different cattle populations. In Britain, the peak incidence of BPP occurs between September and December when the bulk of the calf sales take place. A seasonality of the disease has also been observed in the United States where a high incidence of BPP has been reported during the fall and spring seasons (Frank 1989).

For many years a controversy existed on the role of *P. haemolytica* in the aetio-pathogenesis of BPP. Several workers in North America considered a multifactorial aetiolgy for BPP where many predisposing factors such as primary virus infections, transport-induced stress were thought to be essential for *P. haemolytica* to produce the disease (Rehmtulla and Thomson,1981). These

postulations were refuted following the experimental studies reported by Gibbs *et al.* (1984) in which a disease resembling the field cases was reproduced by intra-tracheal inoculation of the organism alone. Subsequent studies performed by Panciera *et al.* (1984) and Ames *et al.* (1985) in the United States of America were also able to reproduce the disease experimentally in calves following intra-thoracic or intra-tracheal administration of *P. haemolytica* alone.

1.4.2. Virulence Factors of *P. haemolytica*

The virulence of *P. haemolytica* is associated primarily with four virulence factors namely fimbriae, the polysaccharide capsule, leukotoxin and lipopolysaccharide (LPS, endotoxin). The interaction of these virulence factors with components of the pulmonary alveolus is believed to initiate a complex inflammatory reaction resulting in an array of pathophysiological alterations which are manifested as the clinical and pathological changes of bovine pasteurellosis. Apart from the interaction of the virulence factors, each factor on its own plays a specific role in the pathogenesis of the disease. The polysaccharide capsule inhibits both complement-mediated as well as intracellular killing by phagoctes and enhances neutrophil directed migration and adhesion of *P. haemolytica* to the alveolar epithelium (Czuprynski *et al.* 1991). Leukotoxin on the other hand allows the survival of the organism by destroying bovine phagocytic cells and platelets (Clinkenbeart *et al.* 1989a; 1989b; 1989c). The specific role of *P. haemolytica* fimbrae in the pathogenesis of BPP has not been studied but Confer *et al.* (1990) suggested that the fimbrae may enhance colonisation of the upper respiratory tract, the first major step in the production of BPP.

1.4.3. Lipopolysaccharide (LPS)

LPS is a major virulence factor which has wide ranging effects on the host (Confer *et al.* 1990; Fenwick 1990; Whiteley *et al.* 1992). It alters bovine leukocyte function by dose dependent inhibition or augmentation (Confer and Simon 1986) and is toxic to bovine pulmonary endothelium (Paulsen *et al.* 1989;

1990; Breider *et al.*. 1990). Additionally, *P. haemolytica* LPS modifies cardiopulmonary haemodynamics (Keiss *et al.* 1964) and elevates circulatory prostonoids, serotonin, cAMP,and cGMP (Binkhorst *et al.* 1990; Emau *et al.*. 1984; 1986).

Recent studies by Davies *et al.*. (1992) have demonstrated variations in the LPS of virulent and a non-virulent isolates of *P. haemolytica* under different growth conditions *in vitro*. The influence of such differences in the LPS on the virulence of the organism *in vivo* is as yet unknown. *Pasteurella haemolytica* LPS is similar to LPS from other Gram-negative bacteria in which it is an integral component of the outer membrane of bacterial cell wall (Confer *et al.* 1990). Studies conducted by Keiss *et al.* (1964) and Rimsay *et al.* (1981) estimated that the quantity of LPS in dried *Pasteurella haemolytica* cell walls ranges from 12 to 25 per cent and that the majority of the LPS is of the smooth type. The chemical structure of LPS consists of a long chain of sugars (i.e. polysaccharide) and a fat, called lipid A (Riestschel and Brade 1992; ; Lacroix *et al.* 1993). The polysaccharide component of the molecule varies from one bacterial species to another and is made up of the O-specific chain and a two part core referred to as inner and outer core. The O-specific chain is the most variable segment of the molecule and consists of 20 to 40 repeating units that include up to eight sugars. The structure of the inner core (the part linked to the lipid) consists of two unusual sugars one of which is a heptose made up of seven carbon atoms instead of the more typical six carbon atoms. The other is Kdo (3-deoxy-D-manno-2-octulosonic acid) which is found in all LPS molecules and links the polysacchride to the lipid. The structure of the lipid A component of LPS is highly conserved between species of bacteria (Riestschel and Brade 1992) and consists of two glucosamine sugars modified by phosphate and a variable number of fatty acids. Lipid A is the active inflammatory moiety of LPS giving rise to most of the deletrious effects of LPS (Bone 1991).

1.5 Aims of the study

- To develop a sensitive and specific assay which allows accurate quantitation of b-SAA and can be widely used to detect inflammatory conditions in cattle.
- To develop a suitable method to purify b-SAA to provide standard material for the quantification of SAA concentration during APR.
- To identify and characterise the systemic APR in cattle during experimental infection of calves with *P. haemolytica.*
- To compare the systemic APR to different field isolates of *P. haemolytica.*
- To determine whether the APR to purified LPS extracted from field isolates of *P. haemolytica* is capable of inducing an APR similar to that induced by the whole bacterium.
- To identify the cytokines which are responsible for stimulating the acute phase response in bovine pasteurellosis.

CHAPTER II

PURIFICATION OF BOVINE SERUM AMYLOID A

2.1. Introduction

Serum amyloid A (SAA) derives its name from its close biochemical and immunochemical relationship to amyloid A (AA), the fibril protein deposited in tissues in secondary amyloidosis. SAA was discovered approximately 18 years ago when antibodies raised against AA protein were shown to cross-react with a serum component, which was subsequently isolated and identified as consisting of a series of isoforms which are now known as the SAA family (Husby and Natvig 1974; Anders *et al.* 1977; Linke *et al.* 1984). It was found by sequencing that the first 76 amino acids in the N-terminal region of SAA represented the fragment which is deposited as the AA protein in tissues (Linke *et al.* 1975; Benson *et al.* 1985). Several laboratories have been successful in raising antibodies against purified SAA and have used it as a tool to study the site of SAA synthesis (Anders *et al.* 1975; Linke *et al.* 1975). In its native state in serum, more than 90 per cent of SAA in the form known as apo-SAA, is associated with high density lipoprotein$_3$ (HDL/SAA) (d = 1.12-1.21g ml^{-1}) which has a combined molecular weight of 180 kD. In the presence of proteolytic enzymes or on denaturation with acids, alkalis or detergents HDL/SAA releases SAA, a polypeptide of 104 amino acid residues with a molecular weight of 12-14 kD (Benditt *et al.* 1988; Pepys and Baltz 1983; Husebekk *et al.* 1987). In man, a small proportion of apo-SAA is associated with low density lipoprotein (LDL), intermediate density lipoprotein (IDL) and very low density lipoprotein (VLDL) fractions (Feussner and Zeigler 1991).

Serum amyloid A is not a single protein. Heterogeneity of SAA has been reported in several species including man (Eriksen and Benditt 1986; Parmelee *et al.* 1982; Sletten *et al.* 1983; Dwulet *et al.* 1988), rabbit (Tobias *et al.* 1982; Liepnieks *et al.* 1991), mouse (Benditt *et al.* 1979), hamster (Niewold and Tooten 1990), horse (Husebekk *et al.* 1986; Pepys *et al.* 1989; Nunokawa 1992) and monkey (Parks and Rudel 1985). In man, there are 6 isoforms of SAA

(Strachan *et al.* 1989; Raynes and McAdam 1991) while in mice SAA is encoded by at least three genes designated SAA_{1-3} (Benditt and Meek 1989). Niewold and Tooten (1991) demonstrated four isoforms of hamster SAA. In the bovine, only one isoform has been identified up to the present time (Rossevatn *et al.* 1992).

The hepatocyte is believed to be the major site of SAA synthesis and does so upon stimulation by cytokines, particularly IL-1, IL-6 and TNF_{α} (Selinger *et al.* 1980; Husebekk *et al.* 1985; Sipe 1992). SAA synthesis has been detected in extra-hepatic tissues such as gastrointestinal tract, spleen and kidney by identification of SAA-specific mRNA (Sipe 1990).

Although SAA is present in serum primarily during the APR, indicating that it has a role in the host non-specific defence system, its biological function is not yet clear. One proposed function is that HDL/SAA is involved in reverse cholesterol transport by guiding HDL from sites of inflammation to the liver (Glomset 1968). Serum amyloid A can preferentially direct HDL to macrophages (Kisilevsky 1991) and at the site of injury macrophages absorb cholesterol and lipid debris so that the HDL/SAA may aid the transfer of cholesterol from these macrophages to tissues, such as liver, where it can be further metabolised (Kisilevsky 1991; Kisilevsky and Subrahmanyan 1992). A second possible role of SAA in cholesterol metabolism may be as a modulator of lecithin:cholesterol acyl transferase (LCAT) activity on HDL (Kisilevsky 1991). A third possible function of SAA is that it may aid in the removal of bacterial endotoxin, which is known to bind to HDL so that the rapid clearance rate of HDL/SAA may accelerate the elimination of complexes of such toxins or other unwanted tissue debris which can associate with HDL (Tobias *et al.* 1982). Another suggestion is that SAA has an immunoregulatory role as it may induce fibroblast collagenase synthesis (Brinckerhoff *et al.* 1989) while more recent work suggests that SAA may play a role in regulating tissue collagenase gene expression or oxidative burst in neutrophils (Linke *et al.* 1991).

Serum amyloid A has been purified from serum of humans (Benditt *et al.* 1979; Raynes and McAdams 1988; Smith and McDonald 1991), hamsters (Niewold and Tooten 1991), mice (Sipe *et al.* 1989), rabbits (Liepnieks *et al.* 1991) and recently from bovine serum (Rossevatn *et al.* 1992). The purified apo-SAA has been used for amino-acid sequencing (Husebekk *et al.* 1988; Rossevatn *et al.* 1992), to characterise the protein (Benditt *et al.* 1979; Marhaug *et al.* 1988; Niewold and Tooten 1991) and to raise antibodies (Raynes and McAdams 1988) for immunoassay development. Although some laboratories have been successful in raising antibodies against SAA, the human protein is a relatively poor immunogen in rabbits, goats and sheep (McAdam *et al.* 1982; Marhaug *et al.* 1988; Pepys and Baltz 1983).

Different forms of bovine amyloidosis have been reported (Gruys and Timmermans 1979; Hol and Gruys 1984) and AA protein has been purified from these tissue deposits by water extraction and repeated gel filtration in the presence of guanidine-HCl (Hol and Gruys 1984). Furthermore, bovine AA amino-acid residues showed a high homology (87%) to the AA proteins of other species. Human AA, present in secondary amyloidosis cross-reacted strongly with antibodies raised against bovine AA (Gruys and Hol 1984). Indeed, these antibodies have been used by Boosman *et al.* (1989) to develop an ELISA for estimating b-SAA. Rossevatn *et al.* (1992) performed a complete amino-acid sequence of b-SAA following isolation of the protein by sequential ultracentrifugation (Havel *et al.* 1955). According to Rossevatn *et al.* (1992) b-SAA consists of 112 amino acid residues compared to 104 residues present in other species which have been studied. This difference is attributed to the insertion of 9 amino acids between position 69 and 70 of b-SAA. Despite the increased number of amino acid residues there are several common characteristics between b-SAA and the SAA of other species such as human, horse and mink. The C-terminal region and the middle region (residues

33-63) of b-SAA are similar to other species whereas from residues 1 to 32 and from residue 64 to 73 vary from species to species.

As more than 90 per cent of SAA in circulation exists as an apolipoprotein of HDL, methods used for HDL apolipoprotein isolation can be applied to the initial stage of SAA purification. The traditional method for HDL isolation is sequential density gradient ultracentrifugation in which various lipoprotein fractions are separated one step at a time (Havel *et al.* 1955). However, obtaining a significant quantity of SAA by this process is laborious and time consuming. In contrast, hydrophobic interaction chromatography (HIC) used in the purification of human SAA (Raynes and McAdam 1988) and affinity chromatography (AFC) used in the preparation of hamster SAA (Niewold and Tooten 1991) offer convenient alternatives for the initial stage in the isolation of b-SAA.

In the present study, hydrophobic interaction chromatography, affinity chromatography and a single-step method of preparative ultracentrifugation (Liepniek *et al.* 1991) have been used in the initial purification of b-SAA followed by repeated gel filtration for further purification of the protein. These three methods have been evaluated and the purified b-SAA was subsequently used to quantify a pool of APbS to use as assay standards in the development of an ELISA for bovine serum (Chapter III).

2.2. Materials and Methods

Phenyl-Sepharose 4B, molecular weight standards, guanidine-HCl, 3-amino-9-ethyl carbazole (AEC), cholesteryl hemisuccinate agarose, dimethyl formamide, dithioerythritol, Tween-20, bovine serum albumin (BSA), bicinchoninic acid (BCA) protein assay kit (B-9643), cellulose membrane dialysing tubing (12 kD cut-off) and other general laboratory chemicals were obtained from

Sigma Chemical Company (Poole, Dorset, UK). Sephacryl S-200 and Superose-12 beads, for preparation of gel filtration columns, and the fast protein liquid chromatography (FPLC) system were from Pharmacia AB, Pharmacia Ltd., (Milton Keynes, Bucks, UK). Nitrocellulose paper was purchased from Bio-Rad Laboratories Ltd. (Bio-Rad House, Hertfordshire, UK). Horseradish peroxidase-conjugated anti-rabbit IgG was obtained from the Scottish Antibody Production Unit, (Law Hospital, Lanarkshire, UK). Rabbit antiserum to human SAA was obtained from Calbiochem, (Novabiochem, Nottingham, UK Cat. No. 566702). The Chloride Analyser 925 was manufactured by Ciba-Corning Diagnostics Ltd, (Essex, UK). The densitometer was a Chromoscan-3 supplied by Joyce-Loebl Ltd., (Gateshead, Tyne & Wear, NE, UK). Beckman L7 ultracentrifuge and Beckman Ti50.4 rotor was from Beckman Instruments UK Ltd., (Milton Keynes, Bucks, UK).

2.2.1. Purification of b-SAA

The APbS used in this study was harvested from the blood collected at slaughter from a cow with an ulcerating tumour. The serum was separated and stored at -20°C until it was used.

2.2.1.1. Hydrophobic interaction chromatography (HIC): A phenyl-Sepharose 4B column (3x20 cm) was prepared and equilibrated (flow rate 0.2 ml min^{-1}) with 5 column volumes of 10 mM phosphate buffer, pH 7.4 containing 4 M NaCl (PBS). The serum was dialysed overnight against 10 mM phosphate buffer (PB), pH 7.4, and applied to the column in 100 ml aliquots. The column was washed with 500 ml of PBS and then eluted with a reducing chloride gradient going from 4 M NaCl in 10 mM PB to 10 mM PB alone, at a flow rate of 0.5 ml min^{-1} over a total volume of 180 ml. This was followed by an increasing gradient, starting from 10 mM PB and rising to 6 M guanidine-HCl in 10 mM Tris-HCl buffer (pH 7.0) over a total volume of 150 ml at a flow rate of 1.65 ml min^{-1} to elute the bound material. The gradients were formed and controlled with a FPLC

and 1 ml fractions were collected. The eluant was monitored at 280 nm and the chloride concentration of the fractions was determined using a chloride analyser. Ten microlitre aliquots of the fractions were analysed on immuno-dot-blot assay (Section 2.2.6) using rabbit antiserum to human SAA which demonstrated a specific cross reaction with b-SAA. The specificity of the antiserum was investigated as part of the validation of the b-SAA ELISA which used the same antiserum and is described in chapter 3 and is illustrated by the Western blot shown in Figure 3.5. Fractions containing b-SAA were pooled. Five millilitre aliquots of the pooled fractions were dialysed in cellulose membrane tubing (12 kD cut-off) against three changes of 10% acetic acid and subsequently with six changes of distilled water and finally concentrated by dialysis against polyethylene glycol (PEG, 20,000 M_r) or by ultrafiltration using an Amicon YM3 filter, prior to sodium dodecyle sulphate polyacrylamide gel filtration (SDS-PAGE) (Section 2.2.4). The bulk of the pooled SAA rich fraction was concentrated to 10 ml by dialysis against PEG prior to gel filtration (Section 2.2.1.4) with an aliquot prepared for SDS-PAGE as described in Section 2.2.1.4.

2.2.1.2. Affinity chromatography: Ten millilitre aliquots of APbS were applied to a cholesteryl hemisuccinate agarose column (3 x 30 cm) equilibrated with 0.15 M NaCl. The flow rate was 0.4 ml min^{-1} , the eluant was monitored at 280 nm and recovered in 1 ml fractions. The unbound protein, eluting in the break through peak, was pooled as fraction I. Subsequently the column was eluted with 1 M NaCl to remove non-specifically bound material (fraction II) followed by elution with 6 M guanidine-HCl in 50 mM Tris-HCl (pH 7.4) which released the affinity bound material (fraction III). Between runs the column was reconstituted by washing with 1% (v/v) Triton X-100 in distilled water. Fraction III was concentrated as described in section 2.2.1.1 and subjected to gel filtration as described in section 2.2.1.4.

2.2.1.3 Preparative Ultracentrifugation (UC): Acute phase bovine serum was mixed at a ratio of 3.56 g potassium bromide to 10 ml of APbS to raise the non-

protein density of the serum to 1.21 g ml^{-1}. A 6.5 ml aliquot of the serum-KBr mixture was placed in a thermoplastic ultracentrifuge tube (Ultraclear, 13 x 64 mM; Beckman Instruments Inc.). The tubes were capped and centrifuged at 164,000g, 4°C for 48 hours in a fixed angle rotor which had a maximum capacity of 44 tubes (type 50.4 Ti, Optima L-70 preparative ultracentrifuge; Beckman Instruments Inc.). The top layer (1 ml) which contained the SAA rich HDL_3 fraction was aspirated; dialysed against distilled water and lyophilised. The lyophilised products were delipidated at 0°C by mixing with 1 ml of a 3:2 (v/v) mixture of ethanol:ether, washing with anhydrous ether followed by centrifugation and drying in air. The residue was dissolved in 1 ml of 4 M guanidine-HCl containing 0.01 mg of solid dithioerythritol prior to fractionation on a gel filtration column (Section 2.2.1.4.).

2.2.1.4. Gel filtration: Gel filtration was used as a final step in all purification procedures and was performed on the FPLC system. A Sephacryl S-200 column (0.8 x100 cm) was equilibrated with 4 M guanidine-HCl in 50 mM Tris-HCl (pH 7.0) and the SAA enriched fractions from HIC, AFC or UC were applied in 1 ml aliquots and eluted in the same buffer at a flow rate of 0.4 ml min^{-1}. The eluant was monitored at 280 nm and the SAA content of the fractions (1 ml) was estimated by immuno-dot-blotting (Section 2.2.6). Fractions with a high SAA content were rechromatographed on a Superose 12 column (1 x 25 cm) with the same buffer at a flow rate of 0.13 ml min^{-1}. Fractions with SAA were pooled and dialysed, and the purity assessed by SDS-PAGE (Section 2.2.4). Purified SAA, following gel filtration, was also examined by Western blotting as described in Section 2.2.5.

2.2.2. Bicinchoninic Acid (BCA) Protein Assay

Reagents

The reagents for the BCA protein assay were obtained from Sigma Chemicals Company (Poole, Dorset, UK).

Reagent A: 1 g solution bicinchoninate (BCA), 2 g Na_2CO_3, 16 g sodium tartrate, 0.4 g NaOH, and 0.95 g $NaHCO_3$ were brought to 100 ml with distilled H_2O and the pH adjusted to pH 11.25 with 10 M NaOH.

Reagent B: 0.4 g $CuSO_4.5H_2O$ in 10 ml distilled H_2O. Solutios A and B are stable. *Working solution*: The working solution was prepared by mixing 100 volumes of reagent A with 2 volumes of reagent B. The reagent is stable for 1 week and should be green in colour.

Protein standard: Bovine serum albumin (BSA) was prepared at concentrations of 2, 1, 0.5 and 0.25 mg ml^{-1} from a stock solution which was quantified by absorbance at 280 nm based on the known extinction coefficient (ε) for a 1% (w/v) solution of BSA in a 1 cm light path (Bradford 1976).

Extinction coefficient of BSA at 280 nm
$\varepsilon_{280}^{1\%} = 6.6$

Assay Procedure

The BCA assay was performed in microtitre plates using volumes of 1/10 of those described by the manufacturer. Ten microlitres of standard or sample in duplicate was added to microtiter plate wells and mixed with 200 µl of the working solution. After incubation at 37°C for 30 minutes the absorbance was read at 562 nm using an ELISA plate reader and the protein concentration determined by comparison to the standard curve.

2.2.3. Molar Extinction Coefficient of bovine SAA

The molar extinction coefficient ε_{280}^{1M} of bovine SAA was determined on a (Pye-Unicam 8P8-500 UV/vis spectrophotometer). Purified b-SAA was diluted in duplicate to approximately 0.1% (w/v) in 0.1M $NaHCO_3$ (pH 9.6) and the absorbance at 280 nm (A_{280}) was measured (Eriksen and Benditt, 1986). Protein

concentration was determined by the BCA assay and the M_r was determined by comparison to M_r standards on SDS-PAGE. The molar extinction coefficient was calculated as follows where A_{280} represents the absorbance at 280 nm in a 1 cm light path.

$$\varepsilon_{280}^{1M} = \frac{A_{280}}{\text{Molarity}}$$

$$= \frac{A_{280}}{\text{protein conc.}/M_r}$$

$$= \frac{A_{280} \times M_r}{\text{protein conc. } (g l^{-1})}$$

The extinction coefficient of a 1% (w/v) solution of b-SAA was calculated using the formula mentioned below.

$$\varepsilon_{280}^{1\%} = \frac{A_{280} \times 10}{\text{protein conc. } (g l^{-1})}$$

2.2.4. Sodium Sulphate - Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mighty Small electrophoresis tank (Hoeffer Scientific Ltd.) with 12.5 % (w/v) polyacrylamide slab gels (7.3 x 10.2 x 0.75 cm) as described by Laemmli (1970). Detailed description of all the buffers used in this procedure is given in Appendix 2.1. Aliquots of dialysed fractions were mixed in a ratio of 1:1 with treatment buffer

giving a final concentration of in 5% (w/v) 2,3-dihydroxybutane-1,4-dithiol (dithiothrietol) and heated to 100°C in a water bath for 5 min to reduce disulphide bonds. Serum samples were diluted 1:20 in diluted stacking gel buffer (pH 6.8) and then reduced with treatment buffer as described above. Gels were loaded with 10 µl of treated sample and electrophoresis was performed with tank buffer (25 mM Tris; 192 mM glycine; 0.1% (w/v) sodium dodecyl sulphate; pH 8.3) at a constant current of 20 mA per gel until the bromophenol blue dye front reached the bottom of the gel (approximately 1 hour). The gels were then stained for 30 minutes with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid in distilled water and subsequently destained in the same solvent mix. M_r standards, which were bovine albumin, 66 kD; egg albumin, 45 kD; pepsin, 34.7 kD; trypsinogen, 24.1 kD; β lactoglobulin 18.4 kD and lysozyme, 14.3 kD (Sigma MW-SDS-70KIT) were included at a concentration of 0.5 mg ml^{-1}.

2.2.5. Immunoblotting

Immunoblotting after SDS-PAGE (Section 2.2.4) was performed according to Towbin *et al.* (1979). Prior to protein transfer, gels, nitrocellulose sheets, filter papers and pads were equilibrated in transfer buffer (25 mM Tris; 192 mM glycine; 20% (v/v) methanol, pH 8.3) with gentle agitation for 10 minutes. The polyacrylamide gel after SDS-PAGE was placed on a nitrocellulose membrane, sandwiched between filter paper and Scotbrite pads. Protein transfer was carried out using a Bio-Rad transfer apparatus at 12 mA for 1 hour with the cellulose nitrate membrane on the anodal side of the gel. The nitrocellulose sheets were then blocked overnight by incubating in 5% (w/v) dried skimmed milk powder in 50 mM Tris-HCl-buffered saline pH 7.4 (TBS). The first anti-serum was rabbit anti-human SAA and was prepared at a dilution of 1:5000 in TBS containing 1% (w/v) dried skimmed milk powder and 0.05% (v/v) Tween-20 (TBS-Tween-milk). After incubation for 1 hour at room temperature in the diluted first antiserum the membrane was washed three times for 10 minutes in TBS-Tween-milk and then

incubated for 1 hour in donkey anti-rabbit IgG conjugated to horseradish peroxidase at a dilution of 1:1000 in the same buffer. After three further 10 minute washes in the TBS-Tween-milk buffer, the chromogen solution containing 3 amino-9-ethyl carbazole (AEC) was added after being freshly prepared by dissolving 20 mg of AEC in 2.5 ml of dimethyl formamide, mixing with 47.5 ml of 50 mM acetate buffer (pH 5.5) followed by filtration through Whatman No.1 paper and addition of 25 µl of hydrogen peroxide 30% (v/v). The reaction was terminated by washing in distilled water. The membrane was then dried in air.

2.2.6. Immuno-Dot-Blotting

During purification a semi quantitative immuno-dot-blotting method (Hawkes *et al.* 1982) was used to identify SAA in the chromatographic fractions. A strip of nitrocellulose membrane (Bio-Rad) was washed for 10 minutes by gentle agitation with distilled water and dried at room temperature. Ten microliter aliquots of the fractions of HIC, AFC and UC, were applied in duplicate and dried at room temperature. The paper was then blocked with 5% (w/v) dried skimmed milk powder in TBS for 2 hours and treated as the immunoblotts described in Section 2.2.5. Finally the immuno-dots were semi-quantified by densitometric scanning reflection mode at 530 nm.

2.2.7. SAA Concentration:

The SAA concentration in fractions during purification was measured retrospectively by analysing stored aliquots of pooled fractions with the ELISA described in Chapter 3.

2.3. RESULTS

In the purification of b-SAA, either HIC or AFC or UC was used as an initial step followed by gel filtration for further purification of protein. The purification of b-SAA was performed approximately 15 times by HIC and the results are presented by describing typical examples for the three alternative initial stages and then a typical gel filtration stage. During the comparison of the isolation procedures,

a common starting material was used which was pooled APbS with a SAA concentration of approximately 200 μg^{-1}.

2.3.1. Hydrophobic Interaction Chromatography

The elution profile of 100 ml of APbS from the HIC column is shown in Figure 2.1 giving the absorbance at 280 nm and an estimation of the SAA concentration in the fractions given by the immuno dot blot for SAA. After APbS was applied to the column of phenyl Superose a peak of non-bound protein was collected. After the descending gradient of chloride ions was applied, a further peak of weakly bound proteins was eluted. As elution with a rising gradient of guanidine-HCl proceeded, a series of protein peaks eluted and when the chloride ion concentration reached 2 M a peak was eluted where the SAA-rich HDL fraction was identified. The presence of SAA in this fraction (shaded peaks in Fig. 2.1) was identified by immuno-dot-blotting (Fig. 2.2). In SDS-PAGE (Fig. 2.3), the protein of this peak (the lane D) showed major proteins with molecular weights of >66 kD, 23 to 27 kD and with minor proteins of 17 kD and 12.5 kD. The SAA-rich fraction (shaded peak in Fig. 2.1) was then subjected to gel filtration (Section 2.3.4). Typical performance results from HIC were for a yield of 50% and a purification factor of 18 which are given in Table 2.1 to allow comparison to AFC and preparative UC.

2.3.2. Affinity chromatography

When 10 ml of APbS was applied to the cholesteryl hemisuccinate column, non-bound material was eluted in the break through peak by maintaining the eluant as 0.15 M NaCl till the base line absorbance was approched. Additional non-specifically bound protein was eluted with 1M NaCl, but SAA remained bound to the column. Desorption of SAA was achieved by eluting with 6 M guanidine-HCl (Fig. 2.4.) and the high SAA content of this fraction was identified by its strong reaction with anti-SAA antibodies on immuno-dot-blots. The SDS-PAGE gel

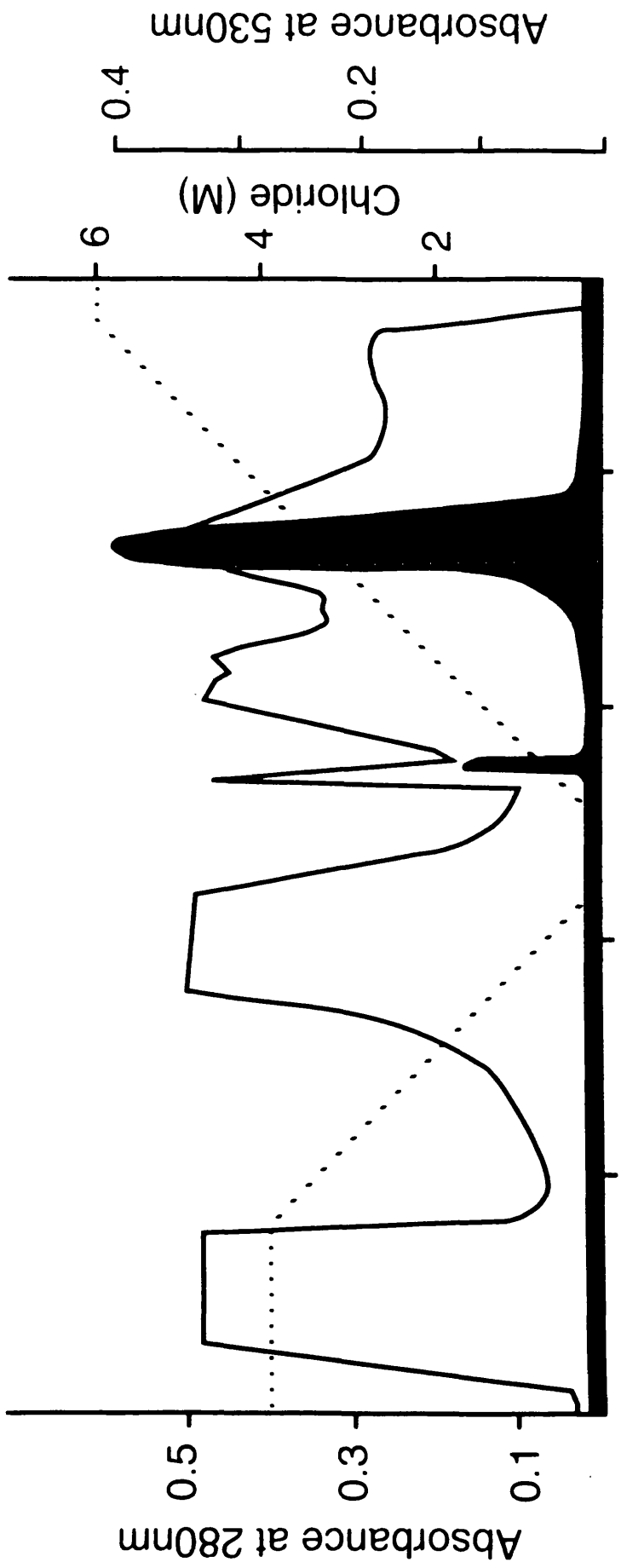


Figure 2.1 Elution profile of acute phase bovine serum (—) obtained by hydrophobic interaction chromatography (HIC). Serum was eluted with a decreasing linear gradient of 10 mM PB containing 4M NaCl to 10 mM PB and then an increasing gradient from 10 mM PB to 6M guanidine-HCl (···). Shaded area represent the fractions containing serum amyloid-A as determined by immuno-dot-blots.

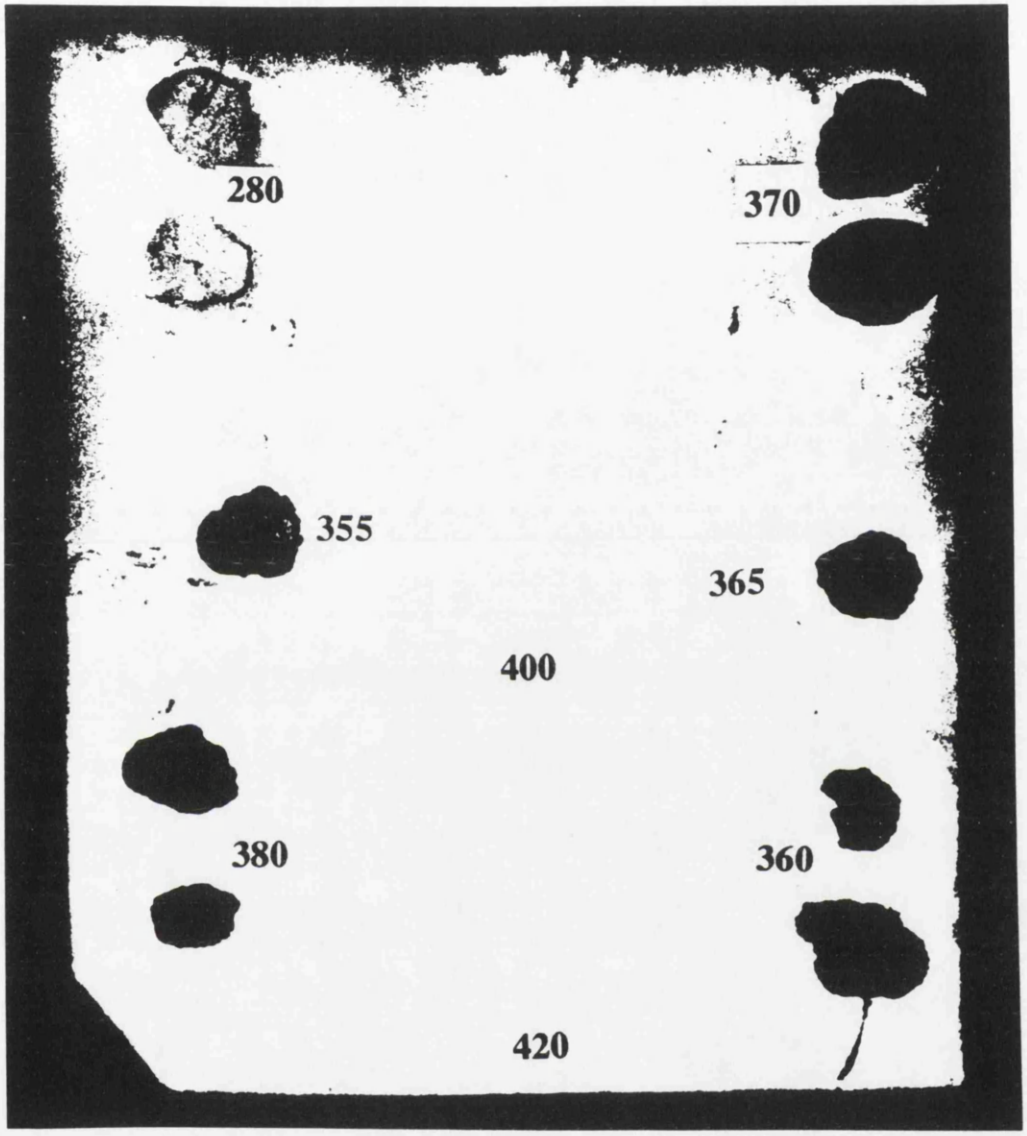


Figure 2.2 Immuno-dot-blots from different fractions from the HIC run shown in Fig. 2.1 and using rabbit antiserum to human SAA as the first antibody. The numbers correspond to the fraction numbers in Fig. 2.1.

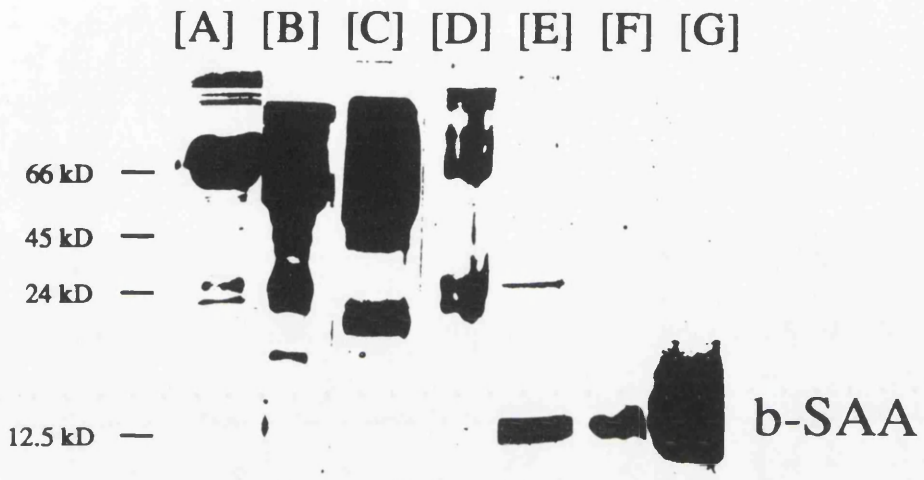


Figure 2.3 SDS-polyacrylamide gel (15%) electrophoresis of (A) Normal bovine serum 1:20 dilution (B) acute phase bovine serum 1:20 dilution (C) fraction C of affinity chromatography (D) b-SAA rich fraction in hydrophobic interaction chromatography (E) b-SAA rich fraction after Sephacryl-200 (F) b-SAA after Superose-12 (G) concentrated purified b-SAA.

(Figure 2.3 [C]) of the SAA rich affinity bound fraction only revealed a faint protein band at M_r of 12.5 kD (i.e. at M_r of SAA) as the fraction was not concentrated sufficiently prior to electrophoresis. Using AFC, the yield of SAA was 45% and the purification factor was 18, which were similar to HIC (Table 2.1).

2.3.3. Ultracentrifugation

After ultracentrifugation of APbS a distinct yellow band was visible on the surface of centrifugation tube contents and this was the total LP fraction HDL which was confirmed as containing SAA by dot blotting. Ultracentrifugation of 6.5 ml of APbS gave 1 ml of HDL rich lipoprotein with a yield of 85% and a purification factor of 87 (Table 2.1). After extensive dialysis against distilled water this fraction was subjected to SDS-PAGE and two distinct protein bands (Fig. 2.5.) were visible with M_r of 23 kD and 12.5 kD. The band with a M_r of 23 kD was likely to be apo-AI.

2.3.4. Gel filtration

Sephacryl S-200 gel filtration of SAA-rich fractions from HIC and AFC gave very similar elution profiles with three main peaks and an example is shown in Fig. 2.6. From ultracentrifugation there were minor differences in the relative sizes of the peaks. The presence of a protein of M_r 12,500 in the third peak was identified by SDS-PAGE (Fig.2.3 [E]) and this peak was positive in the immuno-dot-blott for SAA. Further fractionation of this third peak on an analytical scale FPLC Superose 12 column produced one major peak (Fig. 2.7) which gave a positive reaction in immuno-dot-blot and gave a single band of M_r 12.5 kD on SDS-PAGE (Fig.2.3 [F] and [G] after concentration). Western blotting showed that the rabbit antiserum to human SAA reacted strongly with this protein of M_r 12.5 kD (Fig. 2.8).

As HIC became the method of choice for the initial purification of SAA, the overall performance of the isolation procedure was determined based on a

TABLE 2.1: Comparison of three isolation methods of b-SAA.

Isolation Method	Yield %	Purification Factor
HIC	50%	18
AFC	45%	18
UC	85%	87

HIC, Hydrophobic Interaction Chromatography; AFC, Affinity Chromatography; UC, ultracentrifugation

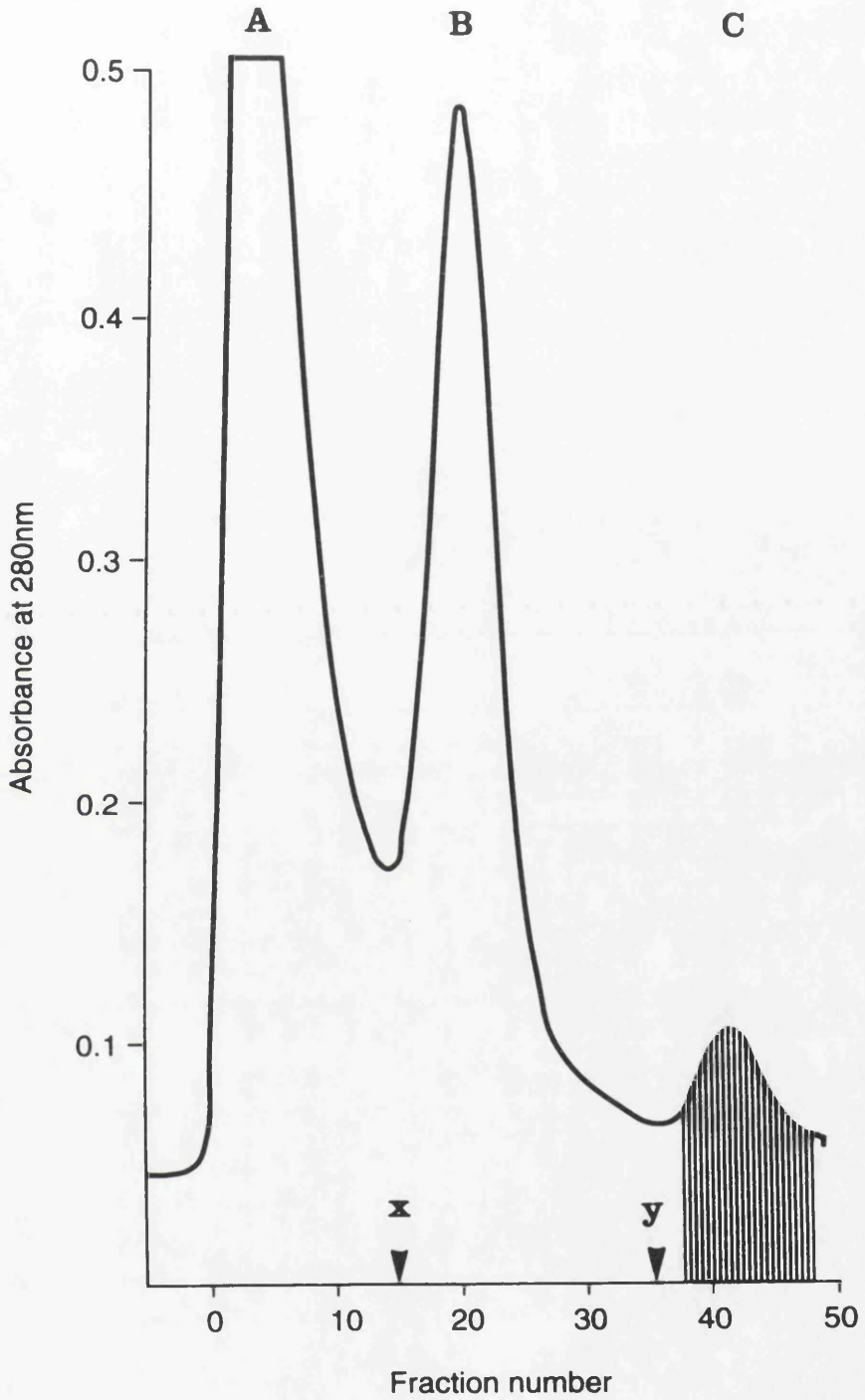


Figure 2.4 The elution profile of acute phase bovine serum obtained by Cholesteryl-hemisuccinate affinity chromatography, sample was applied and eluted at a flow rate of 0.4 ml min^{-1}. Affinity bound proteins were eluted with 6M guanidine-HCl; 0.5M Tris NaCl pH 7.4. Elution was monitored at 280 nm. One millilitre fractions were collected and analysed for b-SAA by dot-blot. Peak A is the unabsorbed proteins; Peak B is non-specifically bound proteins and Peak C is the HDL rich bovine SAA fraction. Arrows show when the buffer was changed to (x) 1 M NaCl and to (y) 6M guanidine hydrochloride. Shaded area shows SAA rich fractions.

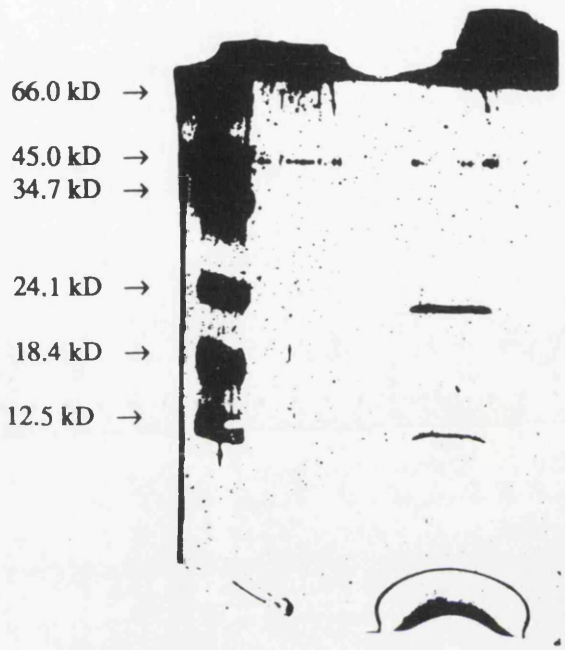


Figure 2.5 SDS-polyacrylamide gel (15%) electrophoresis of the dialysed HDL fraction from the ultracentrifugation and gel filtration on a Sephacryl S-200 column.

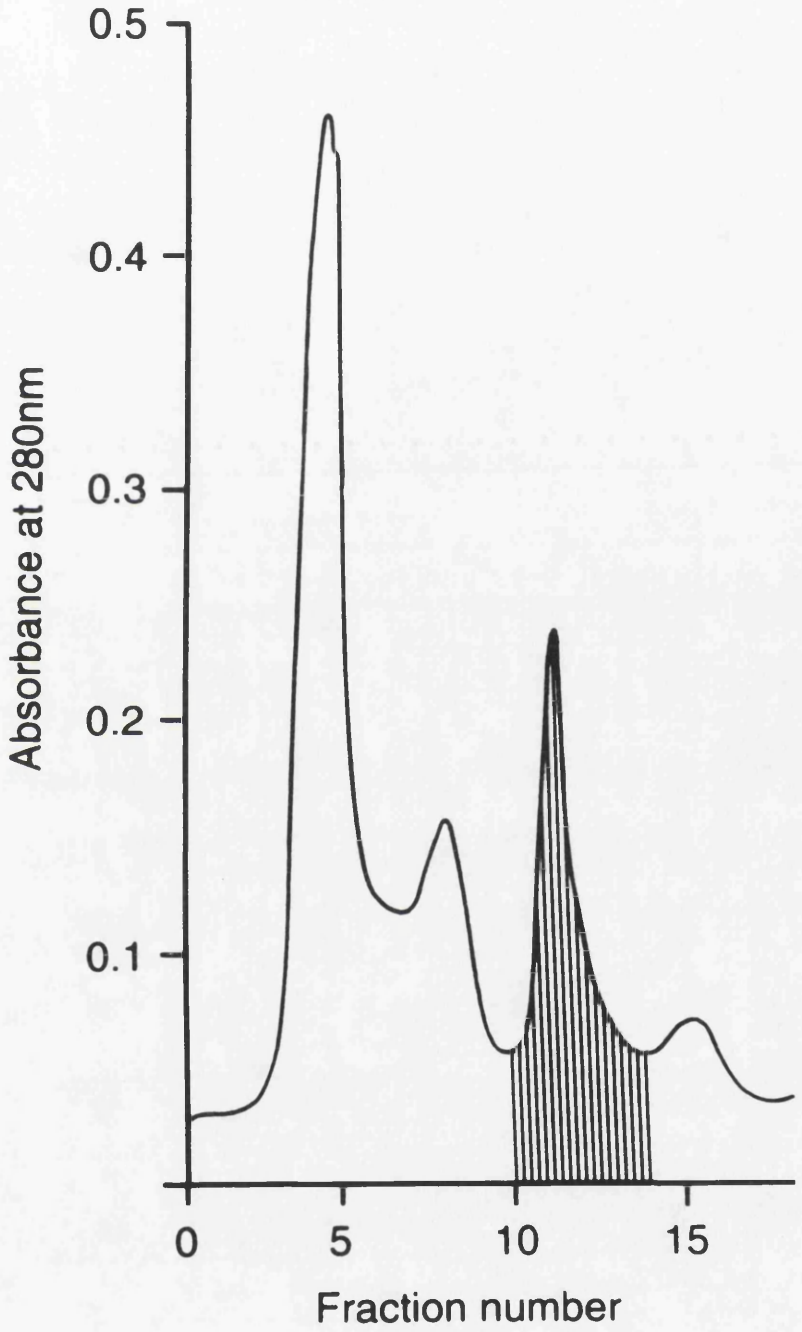


Figure 2.6 Elution profile of Sephacryl S-200 gel filtration chromatography (flow rate 0.2 min^{-1}) with the SAA rich fraction from hydrophobic interaction chromatography. Shaded area represents fractions with SAA.

starting volume of 100 ml of APbS being loaded on to the phenyl Sepharose column. In relation to this starting material the yield of b-SAA after gel filtration on Sephacryl S-200 was 33 % and the purification factor was 80 while after the final Superose-12 column the yield of b-SAA was 25% and the purification factor was 400x (Table 2.2).

2.3.5. Extinction Coefficient of b-SAA

The ε_{280}^{1M} of b-SAA was 22 x 10^3, assuming the M_r of b-SAA to be 12.5 kD as identified by SDS-PAGE, with the absorbance of a solution of 0.34 g l^{-1} being 0.62 OD units. The extinction coefficient of a 1% (w/v) solution of SAA was calculated as 18.

$$A_{280} = 0.62$$

$$\text{Molarity of b-SAA sample} = \frac{0.34}{12.5}$$

$$\varepsilon_{280}^{1M} = \frac{0.62 \times 12.5}{0.34}$$

$$= 22 \times 10^3$$

$$\varepsilon_{280}^{1\%} = \frac{0.62 \times 10}{0.34}$$

$$= 18$$

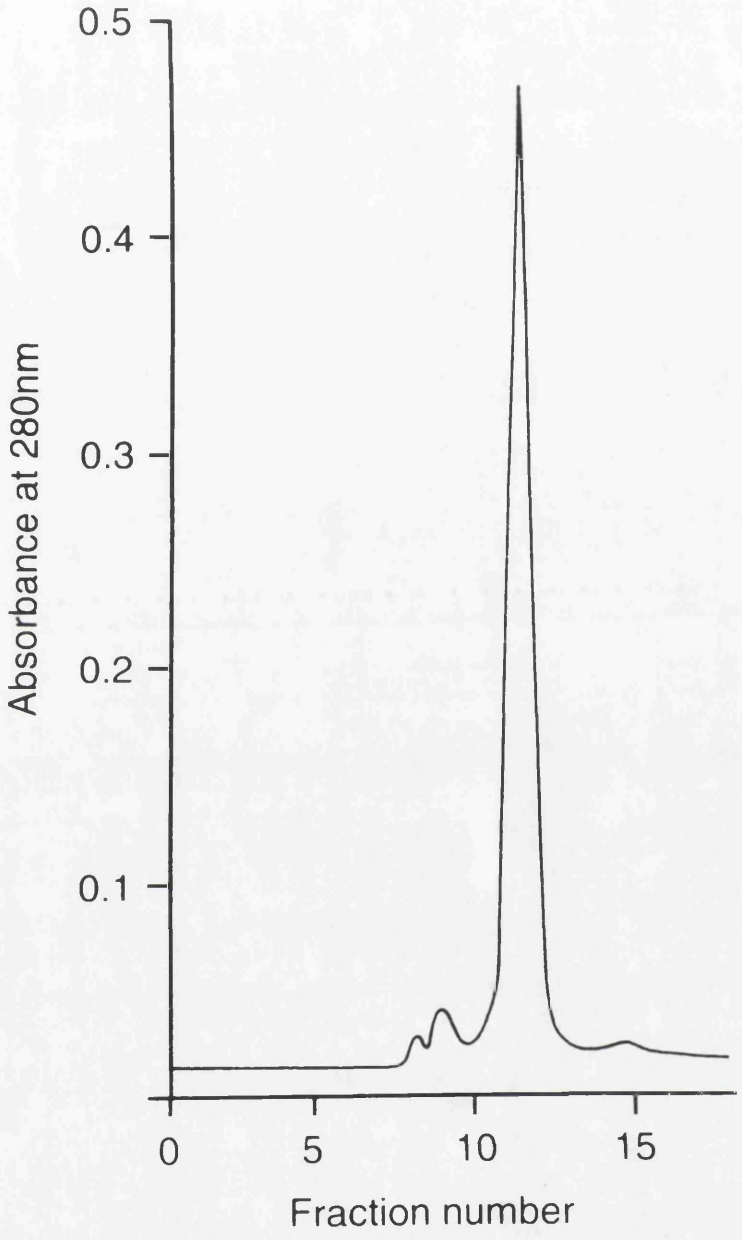


Figure 2.7 Elution profile of Superose-12 gel filtration chromatography on SAA rich fraction from the Sephacryl S-200. Single peak of b-SAA (flow rate 0.2 ml min^{-1} and fraction size 1 ml). The major peak at fraction 11 - 12 gave a positive reaction for SAA in the immuno-dot-blot.

Figure 2.8 Immuno-blott after SDS-PAGE of b-SAA rich fraction from hydrophobic interaction chromatography and gel filtration using rabbit antiserum to human SAA as the first antiserum.

TABLE 2.2: Purification of bovine Serum Amyloid-A

	Volume	Protein. Conc.	Total Protein	SAA	Total SAA	µg SAA	Yield	Purification.
	ml	mg ml^{-1}	mg	µg ml^{-1}	mg	mg^{-1} protein.		Factor
starting matrix APbS	100	72.00	7200	200	20.0	2.77	100%	-
HIC	45	10.00	450	358	15.8	35.8	50%	17
GF-1	10	2.25	10	650	6.5	289	33%	80
GF-2	5	1.00	5	1000	5.0	1000	25%	400

APbS-acute phase bovine serum; HIC- Hydrophobic Interaction Chromatography; GF-Gel Filtration

DISCUSSION

The purification of any protein from a complex biological fluid, such as serum, requires a specific assay system to identify fractions containing the protein. In order to isolate SAA from APbS a suitable detection system was required for the protein. Fortuitously it was found, in the present study, that b-SAA could be detected in column fractions by an immuno-dot-blot method using a commercially available rabbit anti-human SAA, shown to cross react with b-SAA (see chapter 3 Section 3.3.2.1). The use of this antiserum in a Western blot of the 12.5 kD protein isolate by HIC and gel filtration confirmed that b-SAA had been purified by the methods developed during this study.

In the purification based on HIC the isolation of SAA from serum was facilitated by its strong association with HDL which binds avidly to phenyl-Sepharose, providing a convenient first step in the process (Raynes and McAdam 1988). Elution of HDL/SAA was achieved using the denaturing agent guanidine-HCl. The presence of guanidine-HCl at this stage was advantageous as it ensured solubility of SAA and, as the buffer for the final stage of purification by gel filtration also contained guanidine-HCl, it allowed the SAA containing fractions to be applied to the appropriate columns without dialysis. However, small fractions were dialysed against 10% (v/v) acetic acid and distilled water in order to prepare for SDS-PAGE which is readily distorted by high salt concentrations. The SDS-PAGE (Fig.2.3 lane [D]) showed that several other proteins were present. The protein with a M_r of 27 kD, was likely to be apo-AI and the protein with a M_r of 17 kD was likely to be apo-AII as these are the major apo lipoproteins of HDL and have the relevant M_r. Reports of HIC in the isolation of human SAA (Raynes and MacAdams 1988) showed that on a phenyl-Sepharose column apo-AI eluted before SAA while with an octyl-Sepharose column SAA eluted before Apo-AI. Indeed a matrix of octyl-Sepharose gave a better separation between peaks than a phenyl-Sepharose column (Raynes and McAdams 1988) possibly due to the varying hydrophobic interaction of SAA

with different matrices. Better purification might have been achieved with APbS on octyl-Sepharose but there was not sufficient opportunity to compare this matrix with phenyl-Sepharose.

Using HIC a 50% yield of b-SAA was achieved which was slightly lower than the yield of 55% achieved with phenyl-Sepharose in the isolation of human SAA (Raynes and McAdam 1988).

The b-SAA yield obtained with affinity chromatography was similar to that achieved by HIC. This may be due to binding of similar amphipathic regions of SAA in both matrices. In the affinity chromatography method described by Niewold and Tooten (1991), the recovery of HDL_3 in the hamster was 70% while in the same study the density gradient ultracentrifugation method had a recovery of HDL_3 of 80%. However they did not report the recovery of SAA which may not be the same as that of the whole HDL fraction.

Alternative desorbents to guanidine-HCl have been used for elution of bound HDL/SAA from HIC or AFC. However, it has been reported (Anders *et al.* 1975) that murine and human SAA, isolated from acute phase serum by HIC with formic acid dissociating the bound protein for the column followed by gel filtration chromatography in formic acid, may bear little resemblance to apo SAA in its native state in lipoprotein particles. Furthermore, SAA prepared using formic acid is known to be contaminated with other proteins, such as albumin, prealbumin and β2-macroglobulin (Marhaug and Husby 1981; Pepys and Baltze 1983). In another study Smith and McDonald (1991) purified human SAA by HIC using ethanol and 10% formic acid as denaturing agent instead of 6M guanidine. According to these workers the advantages of using ethanol over guanidine was that there was less need for exhaustive dialysis which is required to remove guanidine, before the recovered material can be placed in biological systems. In their method the final recovery after the complete process of purified SAA was 56% which was greater that achieved here or that achieved by Raynes and McAdam (1988).

Wickman (1979), reported the selective binding of HDL to cholesteryl hemisuccinate. The binding of HDL to affinity gel was so strong that, elution was only possible using denaturing agents or detergents, which have to be removed prior to the next stage of purification by extensive dialysis. Niewold and Tooten (1991) purified hamster SAA using the same technique as Wickman (1979), but instead of detergent to denature the bound HDL they used guanidine-HCl. In this study guanidine was found to be a effective dissociating agent in purification of b-SAA by AFC.

Comparison of the performance of the 3 methods demonstrates that HIC and AFC were equivalent to each other but that the single-step UC had a better yield and a higher purification factor. The lower recovery of SAA using HIC and AFC in comparison to UC might result from the fundamental procedures of column chromatography, which present more opportunity to loose material during the process whereas UC took place in one step. Despite this HIC was used for subsequent purification. HIC on phenyl-Sepharose had a major advantage over the traditional method of sequential step UC for lipoprotein in that which used a number of steps, isolating VLDL, LDL and HDL in turn. SAA purification on HIC was more convenient in dealing with large volumes of serum. SAA is present only in mg quantities per litre of serum and large volumes of acute phase serum had to be processed in order to obtain sufficient quantities of b-SAA for future investigation. Preparative UC is time-consuming and laborious and was not appropriate for this purpose. Unfortunately the single-step procedure, described by Liepnieks *et al.* (1991) to isolate SAA-rich HDL from acute serum in rabbits was only available towards the end of this study. In future investigations this is likely to become the method of choice for the initial purification of b-SAA.

Despite the lower yield with the HIC method it is convenient, highly reproducible and is particularly useful for processing of large quantities of serum for apo-SAA purification. The column matrix for HIC is considerably less

expensive than that used in the AFC columns. In the present study therefore, HIC was selected as the method for initial isolation of SAA as it was found to be the more economical of the two column procedures. This study identified the optimal method to isolate b-SAA and it was repeated on several occasions and therefore enabled the development of the quantitate ELISA for b-SAA described in the following Chapter.

CHAPTER III

DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE QUANTIFICATION OF BOVINE SERUM AMYLOID A

3.1. Introduction

The concentration of serum amyloid A (SAA) in plasma is of clinical significance in man (Pepys and Baltz 1983; Sipe 1992) and in cattle (Hol and Gruys 1984; Boosman *et al.* 1989; Gruys *et al.* 1993). There is minimal SAA synthesis during homeostasis, but within a few hours of injury or infection SAA can be detected in the high density lipoprotein (HDL) fraction of plasma (Benditt and Eriksen 1977; Bausserman *et al.* 1980). Among the various biochemical and haematological parameters of the APR in man, SAA exhibits the most striking changes and it is therefore a sensitive marker for monitoring the intensity of APR (Mozes *et al.* 1989). The amount and duration of SAA production during the APR to tissue injury and cell necrosis depends upon the type of injury and its magnitude (McAdam *et al.* 1978; Pepys *et al.* 1989; Nakayama *et al.* 1993). Circulating SAA concentrations may remain elevated for considerable period of time depending on the inflammatory conditions. In viral infections, however, SAA tends to disappear rapidly from the circulation (McAdam *et al.* 1975; Nakayama *et al.* 1993).

Among domesticated species, SAA has been identified as the major acute phase protein in the horse and has shown to be a reliable marker for assessment of tissue damage during training and racing (van Andel *et al.* 1988; Pepys *et al.* 1989). Increased concentrations of SAA are also known to be associated with amyloidosis in the hamster (Niewold *et al.* 1991), rabbit (van Andel *et al.* 1988), dog (Westermark *et al.* 1985), cat (Dibartola and Tarr 1986) and cattle (Gruys 1977; Hol and Gruys 1984). In more recent studies, SAA has been identified as an acute phase reactant in cattle (Boosman *et al.*1989; Alsemgeest *et al.* 1992; Gruys *et al.* 1993). The plasma concentration of SAA increased a 100-fold in response to endotoxin administered intradermally and intravenously (Boosman *et al.* 1989) and SAA was also raised in the peripartum period (Alsemgeest *et al.* 1992). These investigations suggested that monitoring bovine serum amyloid A (b-SAA)

concentration may serve as a specific and reliable marker for clinical inflammation and tissue injury (Gruys *et al.* 1993).

The technical difficulties surrounding accurate and reproducible quantification of SAA concentrations in human plasma and other biological fluids have been widely discussed (Marhaug 1983; Pepys and Baltz 1983; Godenir *et al.* 1985; Benditt *et al.* 1988). It has been reported that optimal immunochemical measurement requires complete dissociation of SAA from the HDL_3 fraction of lipoproteins. Studies have shown that denaturation by heat, acid or alkali increases the immunoreactivity and reproducibility of SAA measurements (Sipe *et al.* 1976; Eriksen and Benditt 1986). In contrast, other investigators report that the use of denaturing treatments results in less satisfactory quantification (Benson and Cohen 1979; Marhaug 1983). The basis for these differing observations is thought to lie in the epitope specificity of the antibodies used and the particular type of immunoassay employed, that is whether radial immunodiffusion, radioimmunoassay or enzyme linked immunosorbent assay (ELISA) were used in a particular study (Sipe *et al.* 1989; McDonald *et al.* 1991).

Immunoassays have been described for the quantitation of SAA in various species. In addition to differences in analytical methods e.g. radioimmunoassay (Rosenthal and Franklin 1975), ELISA (Sipe *et al.* 1989; Pepys *et al.* 1989) or latex agglutination (Yamada *et al.* 1993), these assays have used a variety of sources for their standards with either AA protein, SAA, or acute phase HDL being employed. The antisera used has been either anti-AA (Boosman *et al.* 1989) or anti-SAA and prepared as either monoclonal (Wood *et al.* 1982) or polyclonal antibodies (Godenir *et al.* 1985; Pepys *et al.* 1989; Sipe *et al.* 1989). Recent publications demonstrated the use of latex coated anti-SAA (Yamada *et al.* 1993) and sequence-specific rabbit antibodies in assays for human SAA (Casl and Grubb 1993).

In the studies reported by Boosman *et al.* (1989) the relative concentration of SAA in cattle was estimated by ELISA using a rabbit antiserum to b-AA which required absorption by affinity chromatography to remove non-specific cross reactions. The results were expressed as arbitrarily assigned units based on the percentage of positivity between a negative and positive sample which were foetal bovine serum and a bovine acute phase serum sample, respectively. During attempts to reproduce this assay in our laboratory, it was found that commercially available antisera to human SAA cross reacts specifically with b-SAA. The use of this readily available rabbit polyclonal antibody to human SAA for an ELISA for b-SAA would greatly increase the transferability of b-SAA assays between laboratories (Bangham 1993). A further improvement in the assay for b-SAA would involve quantification with standards of known SAA concentration (in S.I. units such as $\mu g\ ml^{-1}$) rather than by comparison to arbitrary units.

Aims of study

The aims of this study were to develop an ELISA to measure b-SAA using a commercially available rabbit antiserum to human SAA and to quantify the b-SAA in bovine serum by comparison to standards of b-SAA purified as described in Chapter II of this thesis.

3.2. Materials and Methods

3.2.1. Enzyme linked immunosorbent assay

Reagent and Equipment

Reagents were obtained from Sigma Chemical Company Ltd. (Poole, Dorset, UK.) unless otherwise stated. Microtitre plates used were from (Nunc Co., as supplied by Gibco, Paisley, Scotland Cat. No. 439454). An Electric shaker (Luckham Recipro-shake, Luckham Ltd., Sussex, UK.) and an ELISA reader (Titertek Multiskan® Plus, Flow Laboratories, Helsinki, Finland.) connected to a

personal computer (IBM PC) running the ELISA reader programme Immuno Soft (Flow Laboratories) were used in the ELISA assay. The densitometer (Chromoscan 3) was supplied from Joyce Loebl Ltd., (Gateshead, Tyne & Wear, UK.).

Primary antibody

Rabbit antiserum to human SAA was obtained from Calbiochem, Novabiochem, catalogue No. 566702 (Nottingham, UK).

Secondary antiserum

Horseradish peroxidase-conjugated donkey anti-rabbit IgG was obtained from Scottish Antibody Production Unit, Law Hospital, (Lanarkshire, UK).

Coating buffer

10 mM sodium bicarbonate ($NaHCO_3$) and 10 mM sodium carbonate buffer (Na_2CO_3) at pH 9.6.

Assay buffer (PBST)

20 mM mono sodium dihydrogen phosphate NaH_2PO_4, 20 mM di sodium mono hydrogen phosphate Na_2HPO_4, 154 mM sodium chloride NaCl and 0.05% (v/v) Tween-20 (Sigma) buffer at pH 7.4.

Blocking buffer

10 % (w/v), instant dried skimmed milk powder ('Marvel') in assay buffer without Tween-20 (PBS, pH 7.4). The milk powder contained 36.4% (w/w) of protein and 1.5% (w/w) of fat.

Substrate buffer

10 mM sodium acetate (CH_3COONa) buffer with the pH adjusted to pH 5.5, with citric acid.

Peroxidase substrate

25 ml substrate buffer plus 100 µl of 1% (v/v) H_2O_2 plus 400 µl of a stock solution of 0.6% (w/v) 3,3',5,5'-tetramethylbenzidine (TMB) in dimethyl-sulfoxide.

3.2.2 Preparation of bovine SAA standards

To overcome known problems of standardisation of SAA which are caused by its association with HDL_3, working standards were prepared according to the method described for human SAA by Godenir *et al.* (1985) and used subsequently by Sipe *et al.* (1989), McDonald *et al.* (1991) and Yamada *et al.* (1993). The working standards were prepared from a pool of acute phase bovine serum (APbS) in which the b-SAA was quantified by comparison to purified b-SAA in foetal bovine serum (FBS) using SDS-PAGE and densitometry.

Serum amyloid A was isolated by HIC and gel filtration as described in Section 2.2.1.1. After protein determination by the Bicinchoninic assay method (Section 2.2.2), a primary standard of purified b-SAA (1 mg ml^{-1}) was diluted in 1% (v/v) foetal bovine serum (1 ml FBS in 99 ml PBST) at protein concentrations ranging from 3 µg to 200 µg ml^{-1} These primary standards were subjected to SDS-PAGE (Section 2.2.3) along with aliquots of the working standard of APbS at 1:20 dilution. Gels were stained with Coomassie Blue dye and the SAA content in the APbS working standard was determined by comparison to the primary standards by transmission densitometry at 590 nm. The working standard was then diluted in 1% (v/v) FBS in PBST to provide standards over the range of 3-200 µg ml^{-1} of SAA which were stored in aliquots at -20°C. All assays included duplicate wells containing 1% (v/v) FBS in PBST as a blank.

3.2.3 Assay procedure

The ELISA for b-SAA was modified from the assay described by Boosman *et al.* (1989) and the following optimal assay procedure was established after extensive investigation described in Section 3.2.4.

Coating

Standards, controls and test samples were diluted 1:100 in coating buffer and then 100 µl per well was dispensed in duplicate into the wells of a 96 well flat bottom microtitre plate and incubated at room temperature (20-22°C) for 18 hours under constant gentle shaking.

Blocking

After coating the excess sample was removed and the unoccupied binding sites were blocked by the placing 300 µl of 10% (w/v) dried milk in PBS (blocking buffer) in each well and incubating the plate for 1 hour at room temperature (20-22°C) with constant gentle shaking.

Washing

The ELISA plate was decanted and rinsed three times with 300 µl per well of PBST.

First antibody

Rabbit antiserum to human SAA (1:10,000 in PBST) was freshly prepared and 100 µl per well was added after which the plate was incubated for 90 min. at room temperature (22°C) with constant gentle shaking.

Second antibody

After decanting, the plate was washed as above and 100 µl of second antibody, which was horse radish peroxidase conjugated donkey antibody to rabbit IgG (1:2500 in PBST) was dispensed to the wells and incubated as above for a further 90 minutes. Following incubation, excess conjugate was removed by washing as above for four times.

Substrate

The peroxidase absorbed to the wells was detected by addition of 150 µl of freshly prepared peroxidase substrate to each well. The plate was covered with an aluminium foil to prevent photoreactivity and incubated at 37°C for 15 min.

Termination

The peroxidase enzyme reaction was terminated by addition of 50 µl of 2 M H_2SO_4 to each well.

Absorbance

The absorbance was measured at 450 nm using a ELISA plate reader and the results were analysed using the Immuno Soft programme which employed a linear-logarithmic (Lin-Log) transformation in the calculation of the ELISA results.

High, medium and low control samples were included in all assays. The non-specific binding (NSB) was measured in every plate where high control samples with a high concentration of SAA were processed without the first antibody.

3.2.4 Assay optimisation

Assay validation was carried out as described by Sipe *et al.* (1989) in several steps. Each step was carried out minimum of three times, and the mean values are presented in the results (section 3.3).

3.2.4.1 Titration of the first and the second antibodies

Initially, the approximate dilution of the first and the second antisera was determined by coating an ELISA plate with a APbS which was diluted 1:100 in the coating buffer, 100 µl was dispensed to each well and the plate was incubated over night (18 hr.) at 22°C. After decanting, the wells were blocked with 10% (w/v) dried skimmed milk in PBS and washed 3 times with PBST. Serial dilutions of the first antibody (1:1250; 1:2500; 1:5000; 1:10,000; 1:20,000; 1:40,000) and the second antibody (1:1000; 1:2000; 1:4000; 1:8000) were prepared in PBST. The various dilutions of the first and the second antibody were dispensed at the appropriate times in a matrix allowing for every combination of first and second antibody in duplicate. Following this initial screen, the optimal dilutions of antisera were determined by demonstration of the effect of varying antibody

dilutions on ELISA plates on to which the b-SAA standards had been absorbed. A series of assay standards were coated on to six microtitre plates. Three plates were reacted with a constant first antibody concentration (1:10,000) with variation in the second antibody concentration the other three plates had a constant second antibody (1:2500) while the first antibody was varied. The objective was to identify the combination of first and second antibody dilutions which gave a maximum optical density of 1.0-1.5 OD Units for the highest standard and also gave a steep standard curve.

3.2.4.2. Optimisation of the standard and sample dilution

Standard curves prepared by diluting the working standard in foetal bovine serum, for the b-SAA ELISA were obtained by using 3 different dilutions of the standards in the coating buffer. The standards were diluted to 1:200, 1:100 and 1:50 and 100 µl was applied in duplicate to the ELISA plates.

3.2.4.3 Incubation temperatures

The influence of different incubation temperatures on the assay was determined in order to optimise the incubation temperature. Four microtitre plates were coated with two sets of standards and two plates were incubated at 4°C with the two remaining plates at room temperature (22°C). All plates were left overnight with constant agitation and the remaining steps were carried out as described above (Section 3.2.3).

3.2.4.4 Incubation times

The influence of different incubation times with first antibody on the ELISA was determined. Six microtitre plates were coated with standards as described above (Section 3.2.2) and incubated at room temperature overnight. After blocking with dried milk and washing, first antibody (1:10,000 dilution) was added to all the plates which were incubated for different periods. Duplicate plates were incubated

for 30 minutes, for 90 minutes and for 2 hours. All the other steps were carried out as above (Section 3.2.3).

3.2.4.5 Optimisation of assay pH

The standards were coated on to three microtitre plates. Assay buffer (PBST) was prepared with pH values of 8.0, 7.4, 7.2 and 6.8 and used in the. procedure described above (section 3.2.3).

3.2.4.6 Assessment of blocking agents

The ELISA was carried out using 10% (v/v) FBS; 5% (w/v) dried milk; 10% (w/v) dried milk and bovine serum albumin (BSA) 0.1% (w/v) and 0.5% (w/v) as blocking agent in PBS.

3.2.5. Assay Validation

Assay validation was carried out, using methods described by Fraser (1986), Sipe *et al.* (1989), and Price and Newman (1992), on the b-SAA ELISA assay which was performed according to the method described in Section 3.2.3.

3.2.5.1. Assay Specificity

Assay specificity relied on the antibody specificity which was demonstrated by Western blotting (Section 2.2.5). To confirm the specificity of the anti-SAA antiserum, four serum samples (diluted 1:20) which were known to be from animals during the acute phase as they all had a high haptoglobin (Hp) concentration were concentrated by boiling for 5 minutes in treatment buffer and electrophoresed on 15% SDS-PAGE (7.3 x 10.2 cm, 0.75 mM thick) with 30 μg protein per lane (Section 2.2.4.) and duplicate gels were run. After electrophoresis the gels were either stained with Coomassie Blue or Western blotting was performed with the rabbit anti-human SAA antiserum at 1: 500 dilution as first antiserum. The specificity of the rabbit anti-b-AA antiserum (Boosman *et al.* 1989) was also determined in an identical procedure except that the antiserum was used at a dilution of 1:200.

3.2.5.2 Precision

The reproducibility of ELISA was demonstrated by intra-assay and interassay coefficients of variation (CV%). Intra-assay precision was determined by calculating the coefficient of variation (CV) of 25 duplicate samples within one assay using the formula (Fraser 1986).

Interassay precision was estimated by deriving the CV of low, medium and high quality controls (14 $\mu g\ ml^{-1}$, 66 $\mu g\ ml^{-1}$ and 178 $\mu g\ ml^{-1}$) in 25 assays and was calculated by taking the standard deviation as a percentage of the mean of the results from the different assays. The repeatability of the standard curves was analysed by determination of the mean ± SD and CV of the absorbance obtained by the standards over 15 assays.

3.2.5.3 Accuracy

The accuracy of the assay was determined by adding known amounts of purified b-SAA to FBS and then measuring the recovery by ELISA. These samples were mixed by vortexing and allowed to stand for 2 hr at room temperature, vortexed again and then the concentration of SAA determined by ELISA. The recovery was estimated as the observed concentration as a percentage of the expected value.

3.2.5.4 The limit of detection

The limit of detection was determined as the lowest amount of SAA that could be distinguished from the zero standard (Fuentes-Arderiu 1992). This was determined as the SAA concentrations at 2 SD away from the mean (mean ± 2xSD) of the zero standard (1% v/v FBS in PBST).

3.2.5.5 Comparison to other methods

To further confirm the accuracy of the assay, the SAA concentration of six bovine serum samples determined by ELISA was compared to results for the same samples assayed in triplicate by SDS-PAGE followed by densitometry, which is

used in human clinical biochemistry as the reference method for SAA determination (Godenir *et al.* 1985; Yamada *et al.* 1993).

3.2.5.6 Reference range and in vivo validation

Serum samples from 36 clinically healthy calves (12 males, 24 females) present in Glasgow University Veterinary School (GUVS) as experimental animals but sampled prior to any experimental procedure, and from 15 cows at an abattoir were obtained in order to give an indication of the b-SAA concentration in healthy animals. Twenty-five serum samples from cows referred to the GUVS large animal clinic and suffering from a variety of clinical conditions were used to demonstrate the range of concentrations in diseased animals and were compared to the Hp concentration in the same samples in order to compare assay results for these acute phase proteins (McDonald *et al.* 1991; Taktak and Lee 1991).

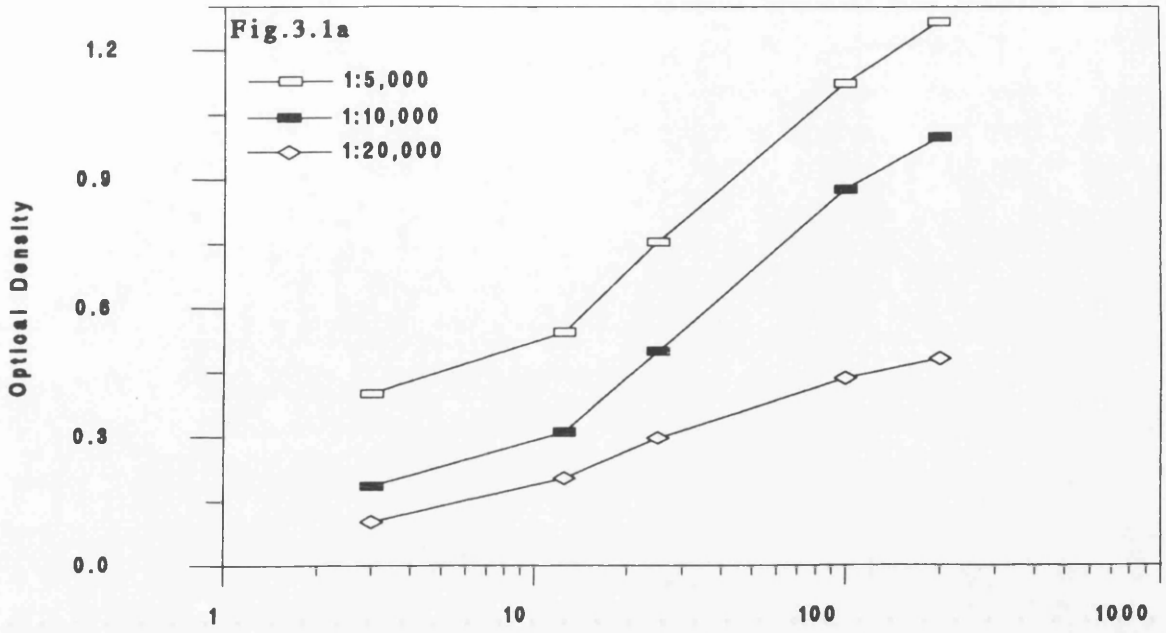
The ability of this SAA ELISA to demonstrate the acute phase response in calves was determined by analysis of the SAA concentration in sera from calves experimentally infected with *P. haemolytica*. However, this was a part of a wider investigation of the acute phase response to *P.haemolytica* in calves which is the major topic described in the next chapter and experimental details are given in Section 4.2.10 and results in Section 4.3.

3.3 Results

3.3.1. Assay optimisation

3.3.1.1 Antibody titration

From the results of the initial screen of antibody dilutions, final optimisation covered a range of 1:5,000 to 1:20,000 of the first antibody (Fig. 3.1a) and a range of dilutions of the second antibody of 1:2500 to 1:4000 (Fig. 3.1b). These studies showed that a combination of 1:10,000 of first antibody and 1:4000 of



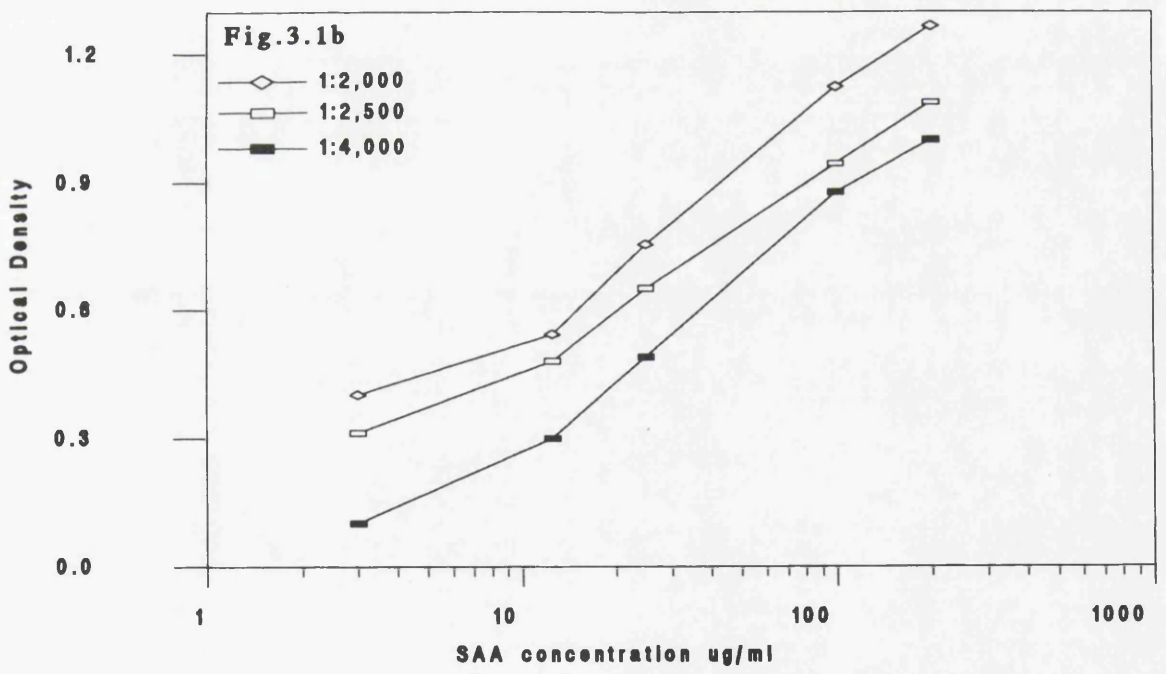


Figure 3.1 Optimisation of the ELISA for bovine serum amyloid A: The effect of the first antibody (rabbit anti-human SAA) dilutions at 1:5000, 1:10,000 and 1:20,000 (Fig 3.1a), and the second antibody (horseradish peroxidase conjugated rabbit IgG) dilutions at 1:2000, 1:2500 and 1:4000 (Fig 3.1b) on the b-SAA standard curve.

second antibody gave the best results with the maximum standard giving an optical density of 1.0-1.5 OD Units.

3.3.1.2 Sample dilution

Figure 3.2a shows the influence of the sample dilution. A dilution of 1:100 gave the best results as with a 1:200 dilution the OD was too low while at a dilution of 1:50 the optical density was too high.

3.3.1.3 Influence of different incubation temperatures on the assay

Incubation temperatures of 4°C and 22°C resulted in similar binding in the assay, and subsequent incubations were performed at 22°C (Fig. 3.2b).

3.3.1.4 Influence of the incubation time on the assay

Figure 3.3a shows the influence of incubation time on b-SAA serum concentration. Thirty minutes of incubation or less was not sufficient to complete the binding as the slope was flat while increasing the incubation time improved binding and increased the slope of the curve. Anti b-SAA binding to the coated well was maximal with an incubation of 1.5-1.8 hours but with 1.8 hours the binding at the low concentrations of SAA increased, reducing the overall gradient of the curve so that an assay incubation time of 1.5 hours was selected.

3.3.1.5 Influence of pH

Figure 3.3b shows the influence of the pH of assay buffer on the b-SAA ELISA. The best results were obtained with pH 7.4 buffer as this gave the steepest standard curve with the OD in the maximum standard being close to 1.0 to 1.5 OD Units. There was little difference with buffers of pH 7.4 and 7.2, and pH 7.4 was selected as the assay pH. At pH 6.8 and 8.0, the standards were flat and giving low binding with the highest standard making other buffers unsuitable.

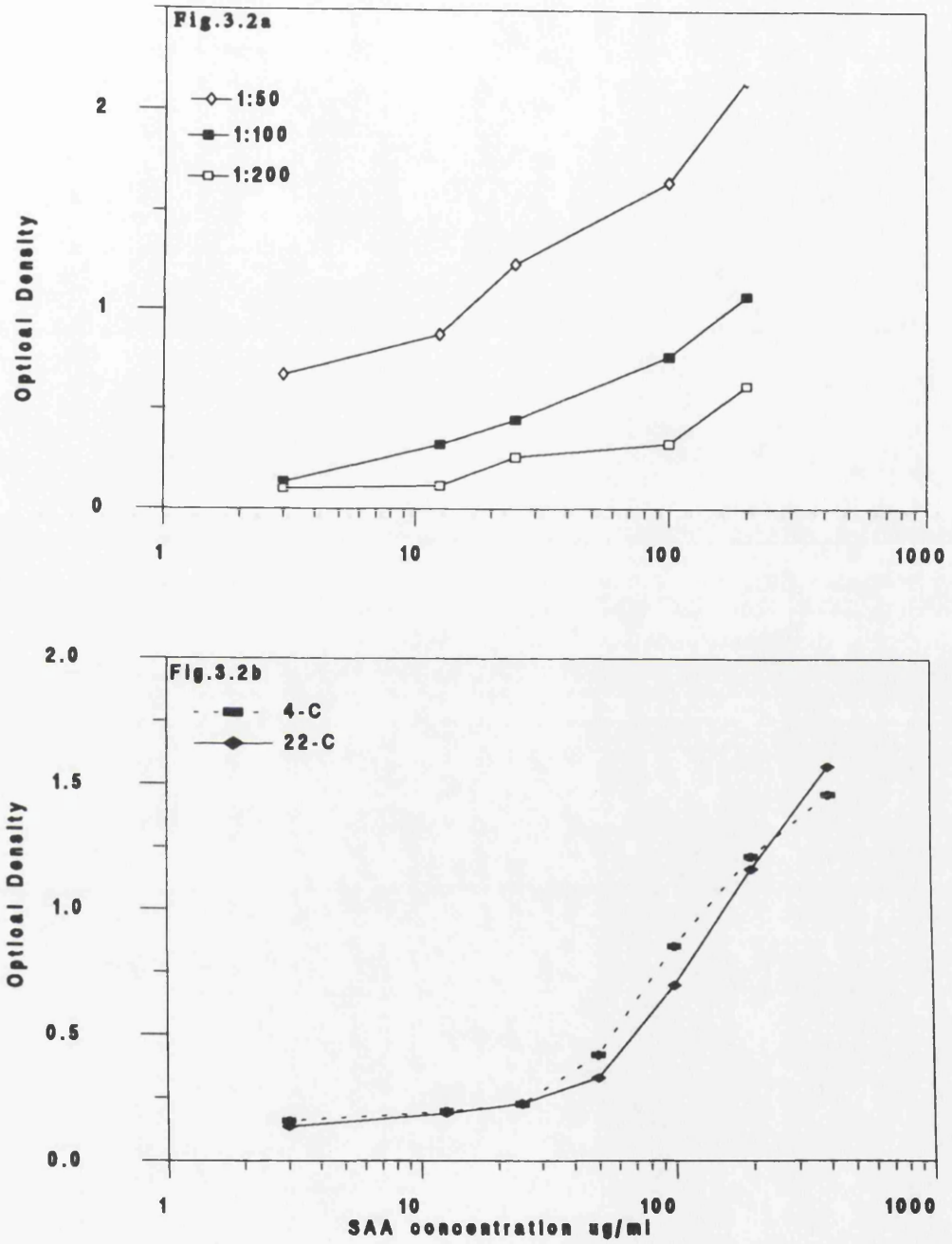


Figure 3.2 Optimisation of the ELISA for bovine serum amyloid A: The effect of sample dilutions at 1:50, 1:100 and 1:200 (Fig. 3.2a) and the incubation temperature at 4°C and 22°C (Fig. 3.2b) on the b-SAA standard curve.

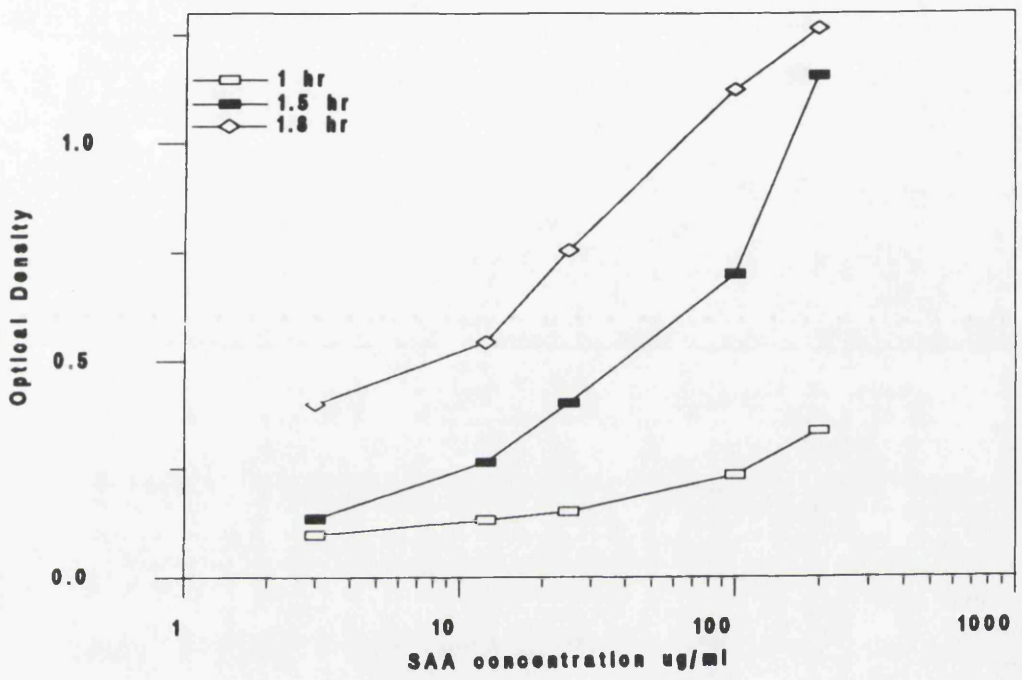


Figure 3.3a Optimisation of the ELISA for bovine serum amyloid A: The effect of the first antibody incubation time at 1 hr, 1.5 hrs and 1.8 hrs on the b-SAA standard curve.

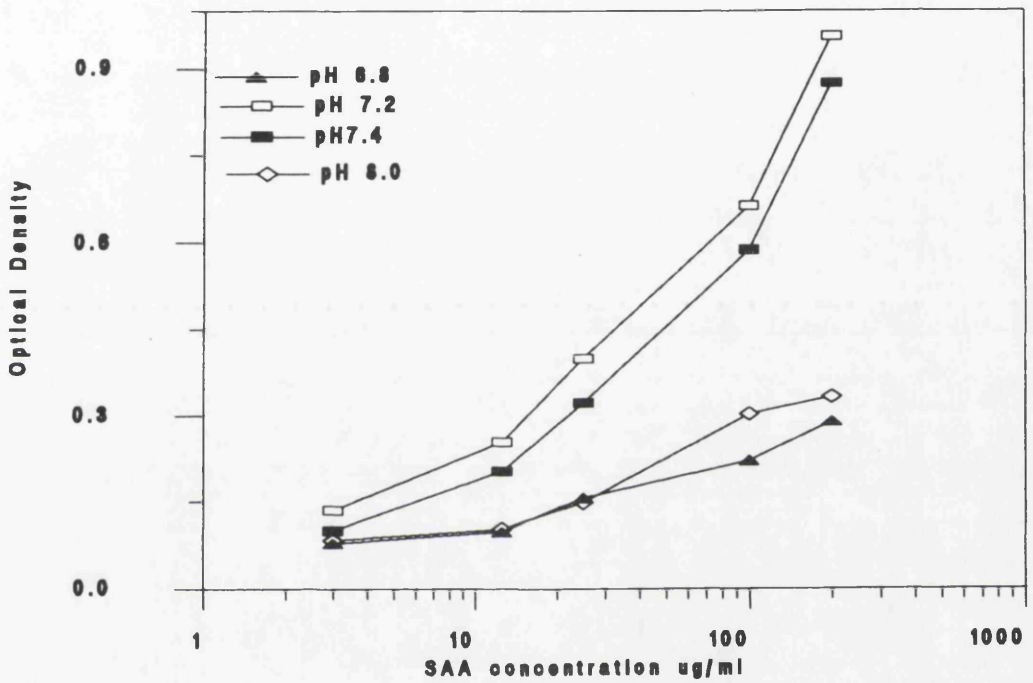


Figure 3.3b Optimisation of the ELISA for bovine serum amyloid A: The effect of the assay buffer pH at 6.8, 7.2, 7.4 , and 8.0 on the b-SAA standard curve.

3.3.1.6 Influence of the blocking agents

Figure 3.4 shows the results of the OD of the zero standard with different blocking agents. The (0.5% w/v) bovine serum albumin (optical density <0.160), foetal bovine serum (optical density <0.050) and 10% milk (optical density <0.060) were all suitable as blocking agents. However, due to the convenience in preparation and lower cost, dried milk of 10% (w/v) was used as the blocking agent in the assay.

3.3.2. Assay validation

3.3.2.1. *Specificity of antiserum to b-SAA*

The rabbit-antiserum to human SAA stained a doublet band in Western blots of APbS (Fig.3.5) which was not evident in Western blots, of normal bovine serum. A similar band stained with antisera to b-AA (Fig. 3.5) but the antiserum was not specific as it reacted with other high M_r proteins. The band seen in Western blots of APbS, which stained with anti human SAA, corresponded to a faint protein band of M_r 12 kD serum in SDS-PAGE gels of APbS stained with Coomassie blue and this band was not present in normal bovine serum (Fig. 3.6).

3.3.2.2. Intra-and interassay precision and the assay repeatability

The intra-assay coefficient of variation was 4.0% for 25 duplicate samples from one assay (Fraser 1986).

The interassay coefficients of variation were 8.8%, 5.5% and 7.2% at b-SAA concentrations of 14 $\mu g\ ml^{-1}$, 66 $\mu g\ ml^{-1}$ and 178 $\mu g\ ml^{-1}$ respectively for 25 assays. The repeatability of b-SAA ELISA standard curve was demonstrated by the CV's of the absorbance at 450 nm of the standards in 15 assays being 5.8 to 11.3% (Table 3.1) and the mean ± SD are shown in Fig 3.7.

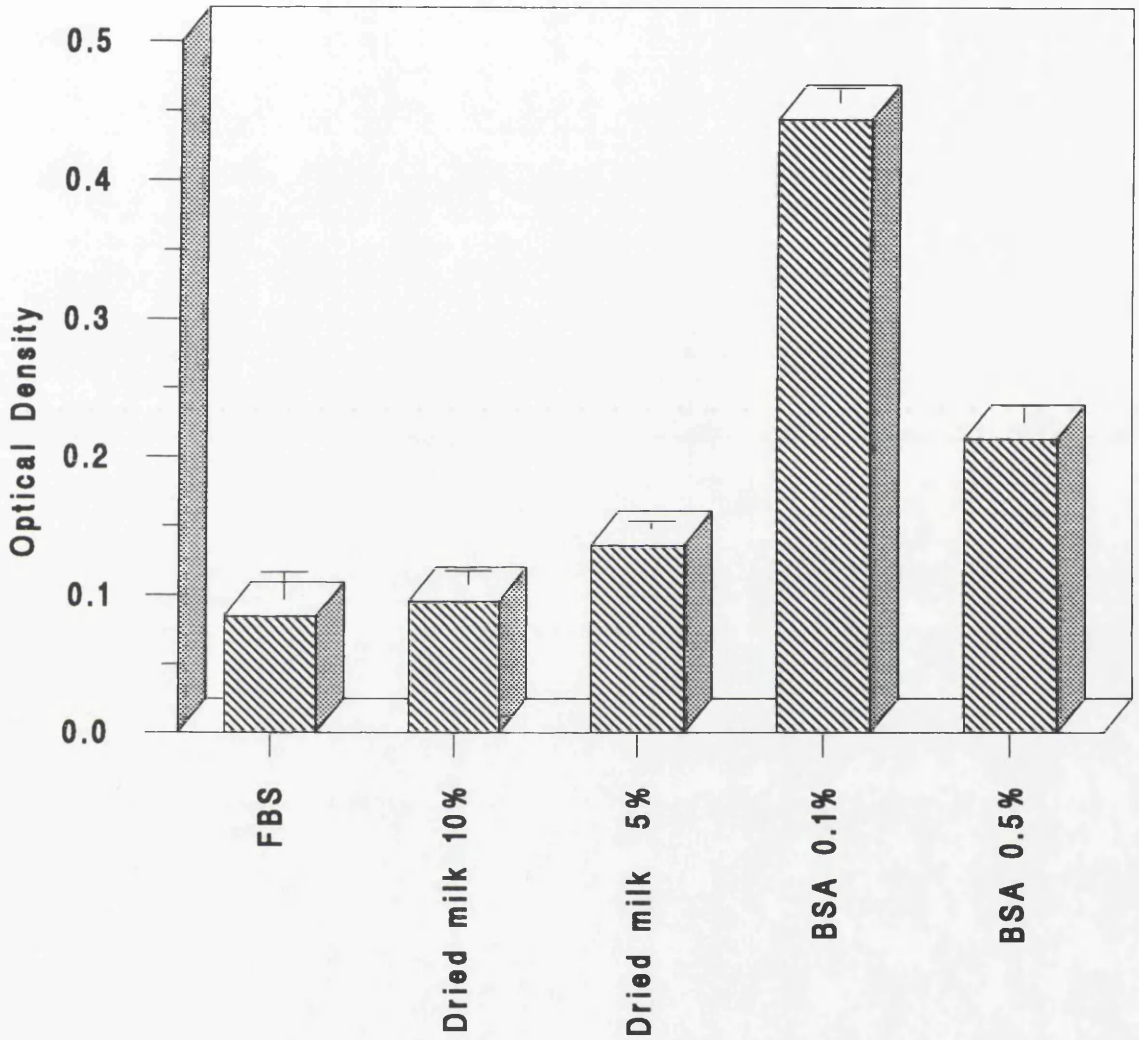


Figure 3.4 Effect of the blocking agents on the b-SAA standard curve. FBS Fetal bovine serum; BSA Bovine serum albumin.

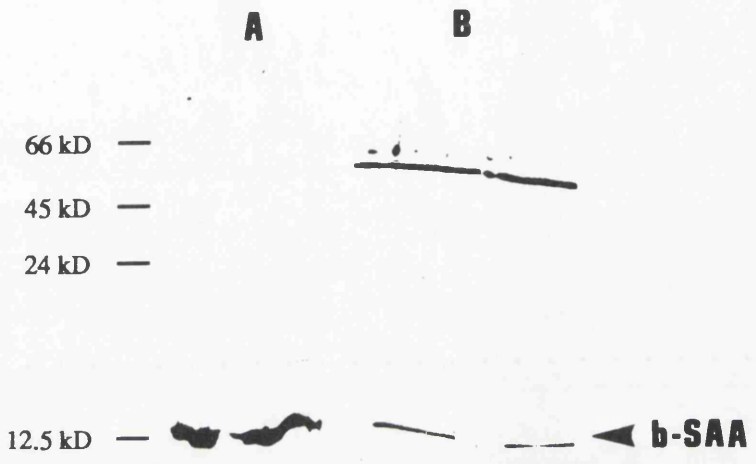


Figure 3.5 Western blots of acute phase bovine serum using rabbit anti-human SAA (A) and rabbit anti-bovine amyloid A (B) as the first antibody.

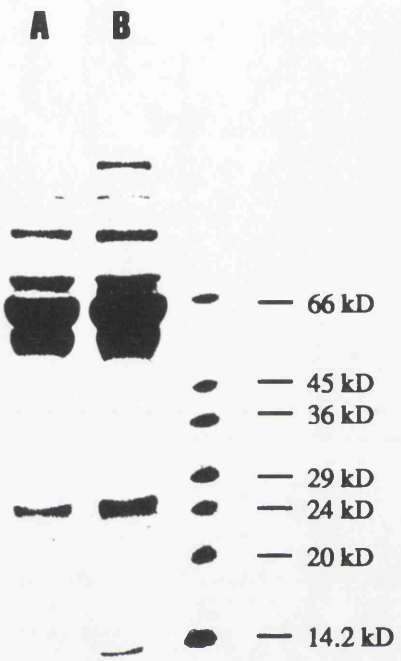


Figure 3.6 SDS polyacrylamide gel (15%) electrophoresis of serum from a healthy calf (A) and an animal with acute pneumonic pasteurellosis (APbS) (B). Note the SAA band with a M_r of approximately 12.5 kDa in the APbS.

Table 3.1. The repeatability of the b-SAA standard curve assessed at the coefficient of variation (CV) of the absorbance at 450 nm of standards in 15 b-SAA ELISA assays.

b-SAA μg ml^{-1}	Absorbance Mean ± SD	CV%
0	0.06 ± 0.1	11.3
3	0.11 ± 0.2	9.6
12.5	0.24 ±0.2	9.2
25	0.30 ± 0.2	8.7
50	0.44 ± 0.1	6.5
100	0.73 ± 0.1	6.1
200	1.11 ± 0.1	5.8

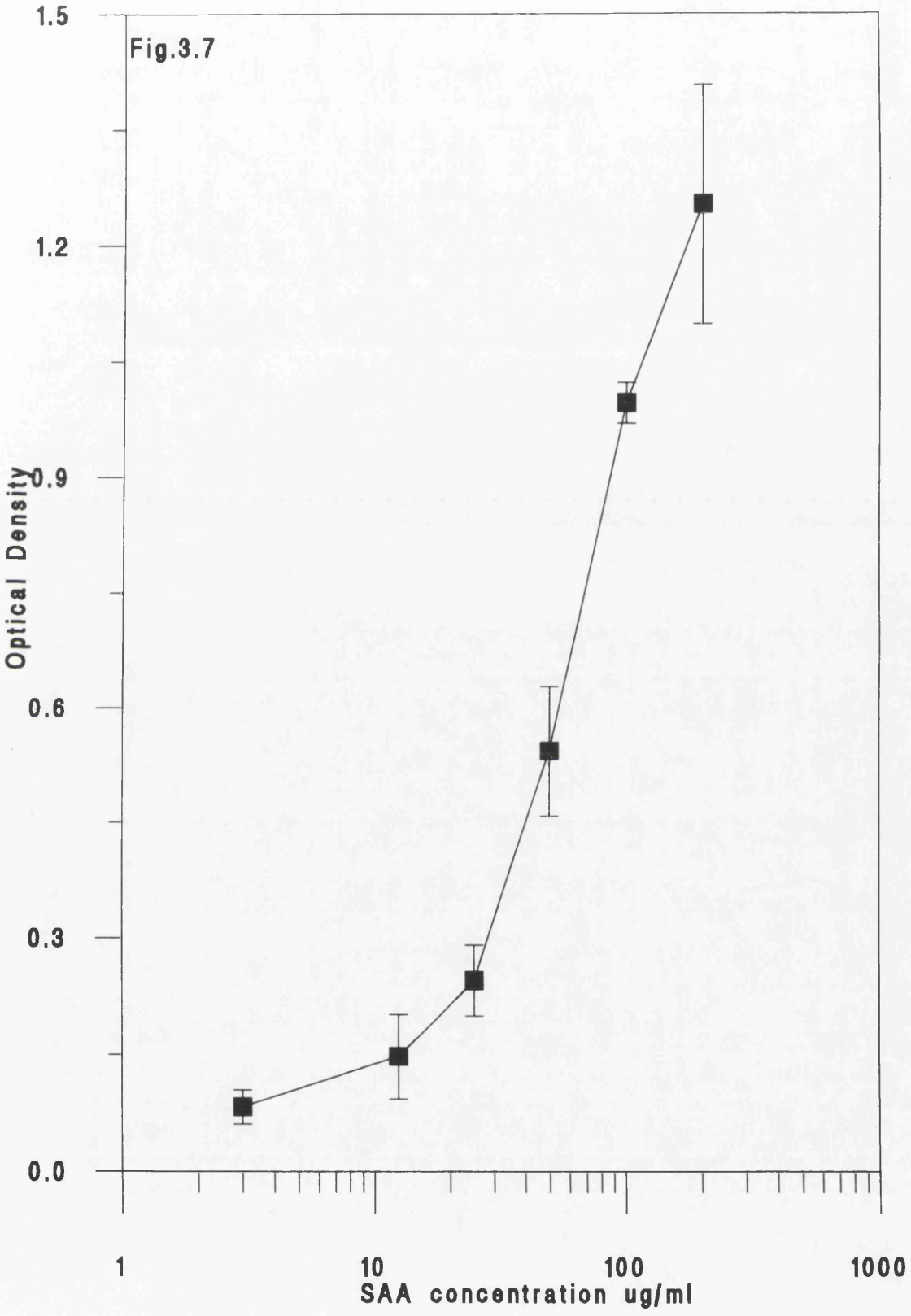


Figure 3.7 Standard curve for quantitation of b-SAA (mean ± SEM).

3.3.2.3. Accuracy

The recovery of b-SAA added to zero serum varied within acceptable ranges of 86 to 112 per cent with a mean of 92 per cent (Table 3.2) The correlation coefficient and linear regression between observed and expected concentrations of b-SAA were calculated. The correlation coefficient (r) was 0.94 ($P<0.05$), and the regression equation was $y = 1.39 + 0.94 x$ where y was the observed and x was the expected concentration of b-SAA.

3.3.2.4. The lower limit of detection

The minimum detection limit significantly different from the zero standard ($P<0.05$) was 0.03 μg of b-SAA in the assay well, representing a b-SAA serum concentration of 3 μg ml^{-1}.

3.3.2.5. Comparison to the reference method

The SAA concentrations of the bovine serum samples measured by both ELISA and SDS-PAGE gave equivalent values (Table 3.3) and the correlation was between results from both methods was $r = 0.94$ $P<0.05$ and the regression equation was $y = -0.55 + 1.05 x$ where y was the b-SAA concentration by ELISA and x was the b-SAA concentration by SDS-PAGE.

3.3.2.6. Clinical validation

The ELISA was used to measure b-SAA concentration in serum samples from animals with different disease conditions and the results are presented in Figure 3.8. The b-SAA in serum samples from cattle with confirmed disease or following surgery ranged from 15 μg ml^{-1} to 130 μg ml^{-1}. Comparison of the b-SAA concentration to Hp concentrations in 25 samples showed that they were significantly correlated $r = 0.58$; $P<0.05$; and with a regression equation of $y= 23.7 + 9.11x$ where y was the SAA concentration and x was the Hp concentration (Fig. 3.9). The b-SAA concentration measured by the ELISA in sequential

Table 3.2 Recoveries of purified b-SAA when added to foetal bovine serum, allowed to stand at room temperature for 2 hours and the SAA concentrations were measured by ELISA. Mean (± SD) was calculated from three separate analyses.

Added SAA μg ml^{-1}	Recovered μg ml^{-1}	Recovery %
170	186 (16.4)	109 ± 8.8%
105	96 (12.2)	92 ± 12.7%
65	56 (6.3)	86 ± 11.3%
25	28 (5.2)	112 ± 18.6%
10	9.3 (3.4)	93 ± 37.0%
5	5.2 (2.0)	104 ± 45.0%

Table 3.3 Comparison of b-SAA concentrations in six bovine serum samples by ELISA and SDS-PAGE analysis results. The table gives the mean ± SD of duplicate estimates.

Sample number	ELISA mean (±SD) μg ml^{-1}	SDS-PAGE mean (±SD) μg ml^{-1}
1	4.8 (0.2)	5.2 (0.3)
2	18.6 (0.9)	20.6 (1.2)
3	88.0 (3.9)	93.2 (4.7)
4	130.0 (5.2)	122.0 (5.3)
5	152.0 (9.1)	166.0 (9.9)
6	178.0 (9.6)	190.0 (9.9)

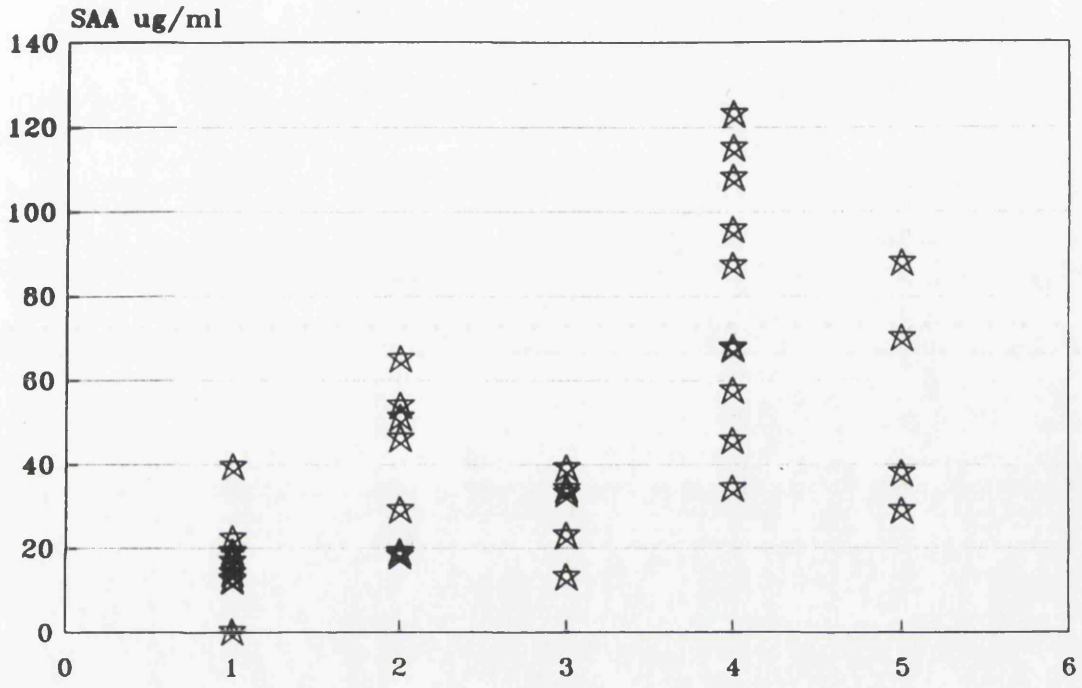


Figure 3.8 Bovine serum amyloid A concentrations in serum from animals with different disease conditions: 1. Unknown field conditons 2. Acute pneumonic pasteurellosis 3. Secondary amyloidosis 4. Liver abscesses 5. Abomasal displacement.

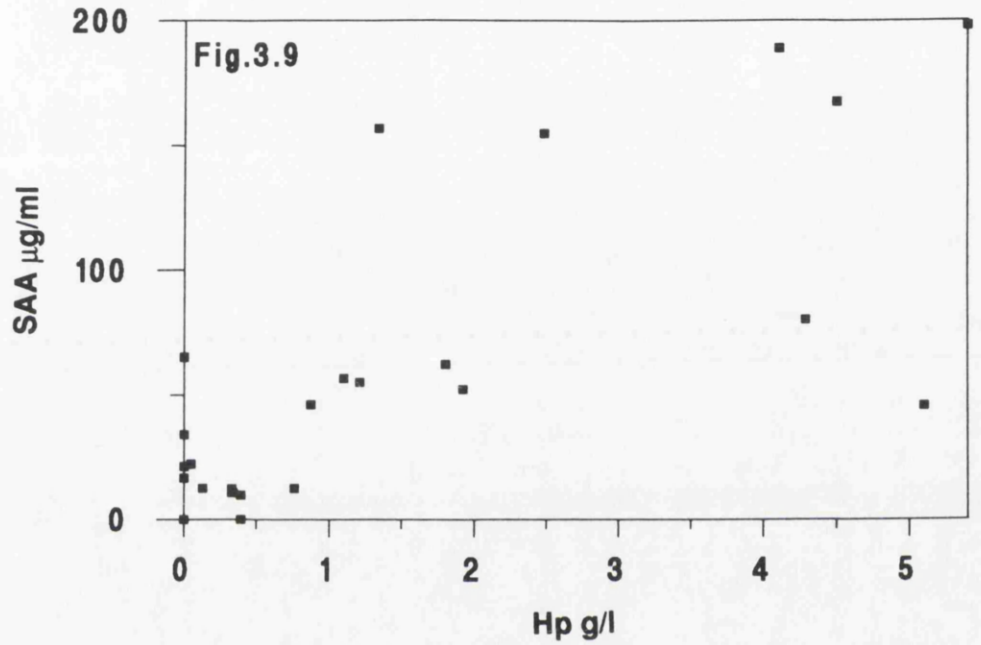


Figure 3.9 Correlation between the 25 parallel measurements of b-SAA and Hp concentrations in cattle (r =0.58; y=23.7 + 9.11x; y =SAA and x =Hp).

samples during the *P. haemolytica* type A1 infection showed a rapid rise to reach a peak within 24 hours and then falling close to baseline levels within 5 days (Fig. 4.6b). This is fully described in the next chapter (section 4.3.8).

3. 4. Discussion

The method described in this chapter is the first ELISA reported to measure b-SAA using a readily available antibody and able to quantitate the concentration of b-SAA in S.I. units. The use of a readily available antibody allows this assay to be more transferable so that it may be performed in laboratories world wide (Bangham 1993). Rabbit anti-human SAA used in the assay was specific to b-SAA and did not cross react with any other proteins when used to stain Western blots acute phase bovine serum. The rabbit anti-bovine AA used by Boosman *et al.* (1989) cross reacted with several other large molecular weight proteins present in serum and an additional absorption step which was reported by Boosman *et al.* (1989) would be required to remove such non-specific binding before use to assay SAA in bovine serum. As the Western blots showed that the antibody to human SAA did not need absorption prior to use, the b-SAA ELISA was developed using it as the first antibody.

The assay of b-SAA in serum does present a problem in that the bulk (90%) of SAA in serum exists in HDL_3 particles with lipid and phospholipid surrounding the hydrophobic portions of the protein (De Beer *et al.* 1982; McAdam *et al.* 1982). Therefore fewer antigenic determinants of SAA are exposed in the native state than when purified SAA is used as standards (Godenir *et al.* 1985; Sipe *et al.* 1989). Serum amyloid-A is also an amphipathic protein, causing problems in solubility and aggregation. The problems of the matrix effect may be minimised by using acute phase serum as the working standard (De Beer *et al.* 1982). In early work on human SAA quantification, results were expressed as arbitrarily assigned units by comparison to positivity of an acute phase serum standard (De Beer *et al.* 1982). However, in subsequent studies (Godenir *et al.*

1985; Sipe *et al.* 1989) purified SAA has been used to calibrate working standards in mass units by densitometry. This technique facilitates the comparison of results between laboratories especially if suitable reference material is available for standardisation.

Boosman *et al.* (1989) in their SAA ELISA for cattle serum used a pool of acute phase HDL to standardised the ELISA and Pepys *et al.* (1989) in horses used a single pool of acute phase horse serum. These were the only assays available to measure SAA in domestic animals so that before the method described here no fully quantitative assay was available to measure SAA in these species. In this study the availability of purified b-SAA allowed the establishment of an ELISA to quantitatively measure b-SAA. Purified SAA, acute phase-HDL and acute phase serum could be used as assay standards. Purified SAA is difficult to solubilise and is unstable in physiological solutions, possibly due to its hydrophobic characteristics, and thus would be unsuitable for use as standard for this ELISA. An APbS sample with SAA concentration determined by comparison to purified SAA on SDS-PAGE was chosen as the most suitable standard material as it shared the same matrix as the samples to be assayed. The same approach has been taken in quantification of non-competitive radioimmunoassay, non-competitive enzyme immunoassay and latex agglutination nephelometric immunoassay for human SAA (Godenir *et al.* 1985; Yamada *et al.* 1989; Yamada *et al.* 1993).

During development of the final assay protocol, described in section 3.2.3, several stages of the assay had to be optimised. Investigations relating to the optimisation of first antibody revealed that the methodology described in this study uses a significantly less volume of rabbit anti-human SAA than does the

procedure of Boosman *et al.* (1989) who used a dilution of 1:1000 of rabbit anti-bovine AA antiserum. The incubation temperature at 4°C and 22°C produced standard curves of a similar shapes therefore in subsequent assays, room temperature was selected as it was more convenient. In the assay described in this study reproducible coating of b-SAA to microtitre wells was achieved with a coating buffer pH of 9.6 as has been described for many ELISA systems (Dubois and Malmendier 1988; Boosman *et al.* 1989; McDonald *et al.* 1991; Taktak and Lee 1991).

Assay validation revealed that the performance of the assay was within acceptable limits for ELISA with an intra-assay CV% of 4% and interassay of less than 9% (Fuentes-Arderiu 1992; Price 1992). These results could not be compared with the previous assay reported by Boosman *et al.* (1989) as they did not present assay validation data. In an ELISA developed to measure human SAA using a commercially available polyclonal antisera the mean intra-assay and inter-assay CV%'s were 7.9% and 7.3% respectively (Taktak and Lee 1991). In another human SAA ELISA developed by McDonald *et al.* (1991) using a rat monoclonal antibody, an average intra-assay CV of 4.9% and inter-assay CV of 7.8% was obtained for four serum samples with SAA concentrations in the range of 74 -1030 $\mu g\ ml^{-1}$.

The ability of the assay to monitor the APR was confirmed by examination of serum from calves before and during experimental infection of *P. haemolytica* and by analysis of normal calf serum and serum from animals suffering from various clinical conditions. Recently Gruys *et al.* (1993) have also observed increases in b-SAA in animals with a variety of conditions including endocarditis, mastitis, pyelonephritis, peritonitis and inflammatory conditions of the gastrointestinal system. In man, SAA concentrations are widely used to detect and monitor the progress of several acute disorders such as myocardial infactions,

renal allocraft rejections and in malignancies (Yamada *et al.* 1993). It is likely that the assay for b-SAA described here, will have an important role to play in detecting the presence of such disease states.

To monitor the time course of the SAA rise during infection, six calves were experimentally infected with *P. haemolytica* type A1. This was part of the experiment described in chapter 4 and full results discussed in that chapter, but the obvious increase of b-SAA concentration following infection as monitored by ELISA (Fig. 4.6b) provides clinical validation of the assay (Fraser 1986). In other words, the ELISA for b-SAA as a marker for the APR in cattle, was able to demonstrate a significant increase in the serum concentration of b-SAA following an experimentally induced APR. The ELISA was shown to offer a simple and sensitive assay to measure b-SAA. The highest concentrations of SAA have been obtained in clinical samples taken in the field, indicating that the response in the experimental animals was not maximal and supporting the potential clinical use of an assay for b-SAA.

The effect of anti inflammatory therapy can be monitored by the measurement of APP (Kushner 1982; Eckersall and Conner 1988; Pepys *et al.* 1989). In cattle, up to the present time Hp has been the most reliable and widely used marker for this purpose (Conner *et al.* 1989; Skinner *et al.* 1991; Kent. 1992) but SAA, due to its greater incremental range could be a more sensitive indicator of inflammation (Boosman *et al.* 1989; Rossevatn *et al.* 1992) and would not be affected by haemolysis which can reduce haptoglobin levels. It has been suggested that SAA and Hp concentrations are regulated independently (Gruys *et al.* 1993), with disease specific pathways involved in their production suggesting a role for b-SAA as a complementary assay for haptoglobin.

In man and certain domestic species SAA is the one other acute phase protein which shares with CRP or Hp the following clinically useful characteristics

of a very low normal plasma level and an extremely rapid change of concentration in response to injury and infection. This extended dynamic range is an important feature of SAA to use as a disease marker. Consequently, in man, CRP and SAA concentrations are used in routine clinical monitoring of inflammation and acute disorders (Kushner and MacKiewicz 1987; Yamada *et al.* 1993). In most human inflammatory diseases, SAA is a more sensitive indicator of inflammatory stimuli than Hp but studies in cattle have been limited due to non availability of a b-SAA assay. The concentration of circulating b-SAA also appeared to correlate with the nature of the disease, whether acute, sub acute or chronic. The quantitative measurements of b-SAA in cattle may therefore be a useful aid in monitoring the course of various diseases.

The main limitation of the b-SAA assay is the assay time and when large numbers of samples are to be analysed a more rapid system would be necessary. In man, a rapid method to measure SAA has been developed recently using a latex agglutination nephelometric immunoassay (Yamada *et al.* 1993). In this system purified anti-human SAA antibody was coated on to latex particles and allowed to react with the SAA standards with the light scattering being read every 15 seconds. After a suitable calibration curve was obtained, 10-100 fold diluted serum samples were processed in the assay. Casl and Grubb (1993) developed another rapid analysis system to measure human SAA with an ELISA using sequence-specific antibodies. The assay time was only 3.5 hours and the working range was 0.1-2500 $\mu g\ ml^{-1}$.

If time and resources are available, the b-SAA ELISA could be improved. For instance purified b-SAA could be coupled to biotin to develop a system where the second antibody is coated to the plate and stored at -20°C prior to use, so that the assay time could be reduced to a couple of hours (Meyer *et al.* 1990). Alternatively the purified analyte could be used to develop a monoclonal

antibodies which could be used in the sandwich format of ELISA; to decrease the limit of detection or reduce the time of the assay.

In summary this assay offers distinct advantages over existing method in that it is accurate, simple, reproducible, sparing of antibodies and can quantitate b-SAA in $\mu g\ ml^{-1}$. Therefore, estimation of b-SAA in serum or plasma will permit more widespread determination of this analyte as a marker of inflammation in both research and clinical situations.

CHAPTER IV

THE ACUTE PHASE RESPONSE IN BOVINE PNEUMONIC PASTEURELLOSIS

4.1. Introduction

Bovine pneumonic pasteurellosis (BPP) is an economically important disease which is manifested usually as a severe acute respiratory disease in calves. Under field conditions several factors such as mixing of herds, environmental changes, transport and stressful management practices precipitate the disease in susceptible animals. Microbiological investigations of the aetiology of BPP have demonstrated the presence of a wide variety of organisms in the respiratory system of affected animals (Yates 1982; Frank 1989). However, in the experimental model developed by Gibbs *et al.* (1984) *Pasteurella haemolytica* biotype A serotype 1 when inoculated intra-tracheally reproduced the clinical, pathological and microbiological features of the disease, which are indistinguishable from the field disease.

The survival of a host challenged by infection or injury depends on many factors. Some are specific, such as antimicrobial T and B cell responses, while others are non-specific. The non-specific host response or the acute phase response (APR) complements the specific responses, commences within hours of the inflammatory stimuli and results in changes in a broad array of cellular, metabolic, endocrine and physiologic functions (Gordon and Koj 1985; Whicher and Evans 1992) and was discussed in detail in Chapter I. The APR may be divided into two phases; a local response which involves coagulation, kinin generation, phospholipid metabolism, vasodilatation and cellular infiltration and a systemic response that encompasses fever, leukocytosis, trace mineral redistribution, hepatic-acute phase protein synthesis, increased stress hormone levels (corticosteroids, glucagon), anorexia and redistribution of body protein and energy substrates (Beisel 1977; Kampschmidt 1984; Dinarello 1984a; Fong *et al.* 1990). The systemic APR helps to inhibit bacterial overgrowth and reduces the degree of tissue damage. Any one component of the APR is insufficient to eliminate a bacterial infection and integration of the systemic response with the

local response may improve the host's condition and ensure survival until a specific immune response to infection occurs.

Despite intensive research on BPP, a clear understanding of the host's non-specific defences against *P. haemolytica* A1 is still lacking (Whiteley *et al.* 1992). Indeed there are few reports of studies on the non-specific response of cattle to bacterial infection but most of these have used *E. coli* endotoxin as a model (Deldar *et al.* 1984; Elsasser 1992). Previous investigations on the host response to pasteurellosis have focused on the local cellular response to *P. haemolytica* A1 (Slocombe *et al.* 1985; 1990; Dalgleish *et al.* 1986; Dalgleish 1989). These investigations have demonstrated an increase in the number of alveolar macrophages and rising neutrophil count in lung lavage collected during the initial phase of clinical disease. Conner *et al.* (1989) examined the changes in the acute phase protein response (APP) during BPP by measuring the concentration of haptoglobin (Hp), ceruloplasmin, seromucoid fraction, α_1 proteinase inhibitor and α_2 macroglobulin. The changes in the APP in this study indicated a rise in serum concentrations of Hp, α_1 proteinase inhibitor and the seromucoid fraction. Haptoglobin which was undetectable prior to the infection, increased within 24 hours and peaked on day 3 and did not return to normal concentrations until day 8. The seromucoid fraction and the α_1 proteinase inhibitor showed a moderate response.

Although some aspects of the APR in BPP namely the cellular response in the lung and the APP changes have been studied, other systemic responses that include endocrine, metabolic, mineral and haematological changes vital for the survival of the host, have not been investigated (Binkhorst *et al.* 1990; Conlon *et al.* 1991). Because BPP is an acute disease the APR is likely to play an important role in protecting the animal, particularly during the initial stages of the infection. Furthermore, an understanding of this complex and multicomponent response may have important implications in the treatment of diverse inflammatory disorders associated with this and other infections. In cattle there has been no thorough and

co-ordinated examination of the many facets of the response therefore findings from this study are likely to be relevant to many other situations where the APR is induced in cattle.

Lipopolysaccharide (LPS) or endotoxin, a component of the outer-cell wall of Gram negative bacteria, has been identified as one of the important components that mediate the virulence of these organisms. The LPS present in the cell wall of *P. haemolytica* A1, is also believed to play a key role in mediating the virulence of this bacterium (Confer *et al.* 1990; Paulsen *et al.* 1989). Recent *in vitro* studies of *Pasteurella haemolytica* A1 recovered from field outbreaks of BPP have demonstrated distinct differences between isolates with regard to their LPS profiles and outer membrane proteins (Davies *et al.* 1992, Ali *et al.* 1992). Although such biochemical variations in field isolates have been recognised, the response of hosts to different isolates of *P. haemolytica* A1 has not been studied prior to this investigation. Indeed it may be that the differences in the structural composition of the bacterial cell wall influence the pathogenicity of the organism *in vivo*. In order to investigate the pathogenicity of different field isolates the pathological lesions in the lung have to be quantified at post-mortem examination. It is possible that monitoring the APR to different field isolates might be an alternative to post-mortem examination, or at least might provide a useful additional means of determination of the pathogenicity of a particular isolate, without euthanasia and post-mortem.

Aim of study

The aim of this study, was to determine the APR to *P. haemolytica* A1 in calves. Thus clinical, haematological and endocrinological changes and variations in the acute phase proteins, trace minerals and clinicochemical analytes in response to *P. haemolytica* A1 infection have been investigated. In addition, systemic APR in calves to two field isolates of *P.haemolytica* A1 were compared in order to determine whether the level of response could be affected by the nature and pathogenicity of the bacterial challenge.

4.2. Materials and Methods

4.2.1. Animals and inoculation procedure

The animals used in these experiments were healthy, weaned, Hereford X Holstein/Freisian calves 2 to 3 months of age with a mean weight of 104 ± 12 kg (range 83-148 kg) and were obtained from a dealer. Calves were selected on the basis of good physical condition, the absence of *Pasteurella* species in the upper respiratory tract as determined from nasopharyngeal swabs obtained 14 and 7 days prior to their arrival at the Glasgow University Veterinary School (GUVS). In addition the animals selected also had a reciprocal serum indirect haemagglutination antibody titre of 8 or less which was considered to be negative for presence of serum antibodies to the organism (Pirie and Allen, 1975). All calves were housed in loose boxes, bedded on straw and fed on hay and concentrates (190 calf pencils, BOCM-Silcock). Group daily food intake was recorded rather than that of individual animals. Water was available *ad libitum* throughout the experiment.

Seventeen calves (12 females and 5 males) were allocated to three groups. Group I and II comprised six calves each and were inoculated intra-tracheally with *P. haemolytica* A1 as described by Dalgleish (1989). In this method a 30 cm stomach tube was introduced through the nose to reach the upper third of the trachea *via* the larynx. A urinary catheter (Arnold Veterinary Products Ltd.) cut approximately to 30 cm, was then passed along the stomach tube and the inoculum was delivered from a syringe which was connected to the catheter.

Animals were inoculated with two field isolates of *P. haemolytica* A1, namely SB 82/1 and SC 82/1. The freezed dried isolates were grown on Trypticase Soy Broth (BBL; Becton Dickson, USA.) at 37°C and a log phase culture was inoculated into calves. All calves were inoculated with 20 ml of a three hour log phase culture of the organism at 11.00 hours followed by 10 ml of a three hour log phase culture at 16.00 hours on day zero. The approximate bacterial counts of these inocula were 1.3×10^{12} and 5×10^{12} colony forming units

(cfu) ml^{-1}, respectively. The inoculates were kindly provided by Dr. H.A. Gibbs, Department of Veterinary Medicine or Dr. R. L. Davies, Department of Microbiology, University of Glasgow . The inoculations were performed by Dr. H.A. Gibbs.

Group III (control group) comprised of five calves. To avoid cross-infection of the control calves, this group was brought to the GUVS at a separate time from the experimental groups. These calves were inoculated with 20 ml and 10 ml of sterile 0.01 M phosphate-buffered saline pH 7.4; 37°C (PBS) intra-tracheally using an identical inoculation procedure to the experimental groups. A summary of the analysis is given in Table 4.1.

4.2.2. Clinical examination

A thorough clinical examination was performed once daily for 3 days prior to infection at 10.00 hours and twice daily thereafter at 10.00 hours and 16.00 hours. On each instance, the following parameters were assessed; demeanour by recording dullness scores (Appedix 4.0), rectal temperature, respiratory rate and character, the presence of cough, nasal and ocular discharges.

4.2.3. Blood samples

Jugular blood samples were collected at 10.00 hours and 16.00 hours using vacutainer tubes containing lithium heparin (10 ml) for acute phase protein determination and 7 ml plain vacutainer tubes fitted with a zinc-free stopper for mineral analysis. Serum was transferred into polyvinyl storage tubes with a plastic pipette. Plasma and serum harvested from blood was stored at - 20°C until assayed. Blood for haematological studies was collected into glass vacutainers (7 ml) containing ethylenediamine tetra-acetic acid (EDTA). All vacutainer tubes were obtained from Becton Dickinson Ltd., (Oxford, UK.).

Table 4.1 Summary of the parameters analysed

	Group I	Group II	Control
Isolates	SB/82/1	SC/82/1	0.01M PBS
No. of calves	6	6	5
Clinical examination	+	+	+
Food intake	+	+	-
Body weight	+	+	-
Haematology	-	+	+
Trace minerals	+	-	+
Cortisol	+	+	+
Thyroxine	+	+	+
Haptoglobin	+	+	+
Serum amyloid A	+	+	+
Ceruloplasmin	+	+	-
Fibrinogen	-	+	+
Clinical chemistry	+	-	+
Post-mortem lesions	+	+	+

4.2.4. Haematological examination

Haematological analysis was carried out at the haematology laboratory, Department of Veterinary Pathology, GUVS. Red cell count (RBC), white cell count (WBC), haemoglobin (Hb) haematocrit (Hct) were assayed in an automated haematology analyser (Roche ABX Minos Vet, Roche Products Ltd, (Welwyn Garden City, Hertfordshire, UK.). The RBC indices mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and the mean corpuscular volume (MCV) were calculated using the Hct, total RBC and Hb content (Dacie and Lewis 1984). Differential cell counts were obtained by examining 200 white blood cells on blood smears stained with May-Grunwald Giemsa-stain.

4.2.5. Analysis of copper, zinc and iron

Serum copper, zinc and iron were measured at the Department of Animal Husbandry of the GUVS by using an atomic absorption spectrophotometer with a Perkin-Elmer analyser (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, UK.). Control serum samples were Precinorm-U from Boehring Mannheim (Lewis, Surrey, UK.). For each mineral a single element bulb with a hollow cathode was used.

4.2.5.1 Copper

Copper was estimated in diluted serum (1:10 in 10 mM Nitric acid) according to the method described by Parker *et al.* (1967) with minor modifications. The atomic absorption spectrophotometer was calibrated for absorbance at a wavelength of 324.8 nm with two copper standards at 1.0 $\mu g\ ml^{-1}$ (equivalent to 15.7 $\mu mol\ l^{-1}$) and at 3.0 $\mu g\ ml^{-1}$ (equivalent to 47.2 $\mu mol\ l^{-1}$). All samples, including the control (a serum sample of Precinorm-U containing copper at 4.0 $\mu g\ ml^{-1}$), were assayed in duplicate and the results were calculated using a HGA-400 Programmer (2380 AAS, Perkin Elmer Ltd.).

4.2.5.2 Zinc

Serum zinc concentrations were assayed using a method described by Butrimovitz and Purdy (1977). The analysis was performed against zinc standards containing 1 $\mu g\ ml^{-1}$ (equivalent to 15.30 $\mu mol\ l^{-1}$) and 3 $\mu g\ ml^{-1}$ (equivalent to 45.90 $\mu mol\ l^{-1}$) prepared in glycerol to approximate the viscosity of diluted samples at a wave length of 213.9 nm. The control and the test samples were run in duplicate in the atomic absorption spectrophotometer, and the average values were calculated using a HGA-400 Programmer (2380 AAS, Perkin Elmer Ltd.).

4.2.5.3 Iron

Samples for iron determination were diluted 1:2 with 20% trichloroacetic acid (TCA) solution and heated in a water bath at 90°C for 15 minutes. This procedure precipitated plasma proteins and released iron present in transferrin. The tubes were then centrifuged and the supernatant was directly analysed using the atomic absorption spectrophotometer at a wavelength of 248.3 nm calibrated with a standard of iron at a 5.0 $\mu g\ ml^{-1}$ (equivalent to 89.53 $\mu mol\ l^{-1}$). Haemolysed samples were excluded from the analysis.

4.2.6. Cortisol Radioimmunoassay

4.2.6.1 Assay Design

A solid phase, competitive radioimmunoassay, developed for measuring cortisol in human serum (McConway and Chapman 1986), was modified for use with bovine plasma. In this assay, cortisol in the standards, controls and test samples competed with ^{125}I-labelled cortisol for binding to a suspension of antibody linked to a solid phase. At equilibrium, the solid phase was separated by centrifugation, washed and counted in a gamma scintillation counter (Packard auto-gamma-5230, Pangbourne, Berks, UK). Unknowns were determined by reference to the inverse relationship between plasma cortisol concentration and radioactivity counts.

Reagents

Cortisol radiolabelled with iodine [^{125}I] (cortisol-3-(*o*-carboxymethyl) oximino-2-[^{125}I] iodohistamine) was prepared according to the method described by McConway and Chapman (1986) and was either supplied by Dr. C. Gray, Institute of Biochemistry, Royal Infirmary, Glasgow or purchased from Amersham International plc, (Amersham, Bucks, UK.). Cortisol for the preparation of standards was obtained from BDH (Poole, UK). Sheep anti-cortisol antiserum (S004-201) was provided by the Scottish Antibody Production Unit (SAPU) (Carluke, Lanarkshire, UK.). 8-Anilino-1-naphthalene sulphonic acid (ANS), an inhibitor of cortisol-binding globulin, gelatine, micro particulate cellulose (Sigmacell Type 20), caprylic acid (99% w/v) and carbonyl di-imidazole were purchased from Sigma Chemicals (Poole, Dorset, UK.). Assay buffer was 0.1M phosphate buffer, 0.1% (w/v) gelatine, pH 7.4 and included ANS at 1 % (w/v) when necessary (see Appendix 4.a).

Standards

To improve the limit of detection of the assay an extra standard was included at the lowest end to give 3.91 nmol l^{-1} as the lowest standard in the bovine cortisol assay and the standards ranged from 3.91 to 500 nmol l^{-1}. The eight cortisol standards (3.91, 7.81, 15.63, 31.25, 62.5, 125, 250 and 500 nmol l^{-1}) were prepared in a normal bovine serum pool from which cortisol had been removed by stripping with agarose-coated charcoal as described by McConway and Chapman (1986). Briefly the appropriate amount of stock cortisol (320 mmol l^{-1}) solution (11.832 mg of cortisol dissolved in 100 ml of ethanol) was dried in glass test tubes and mixed with the stripped serum for an hour. The stripped serum was included in the assay as a zero cortisol serum standard. All standards were stored in aliquots at -20°C.

Preparation of anti -cortisol IgG

IgG was separated from sheep anti-cortisol antiserum for the preparation of the solid phase reagent, using a method described by Steinbuch and Audran (1969).

Specificity of this antibody has been described by Naylor *et al.* (1990). Briefly, the pH of the serum (20 ml) was adjusted to pH 5.0 with 0.1 M acetic acid and 1.76 ml of caprylic acid added dropwise. In this process all the proteins except IgG were precipitated. After centrifuging at 1500g for 20 min, the IgG supernatant was separated, collected and then dialysed against three changes of 154 mM NaCl and finally concentrated by dialysis against polyethylene glycol (PEG 20,000 M_r). The protein concentration of the IgG sample was measured by the BCA (Bicinchoninic acid) protein assay (Section. 2.2.2.).

Activation of microcrystalline cellulose

Activation of cellulose and the covalent linkage of IgG was performed as described by McConway and Chapman (1986). In a 50 ml conical flask 5 g of microparticulate cellulose, 0.61g of carbonyl di-imidazole and 25 ml acetone were mixed. This mixture was stirred for 60 min at room temperature to activate the cellulose. The activated cellulose was recovered by filtering under reduced pressure. It was then washed 3 times with 100 ml aliquots of acetone and air-dried on filter papers.

Coupling of IgG to activated cellulose

Coupling was carried out by rotating 1 g of activated cellulose in 4 ml of 0.05M barbitone buffer (pH 8) for 18 hours at room temperature with 500 µl of IgG with a protein concentration of 10.25 mg ml^{-1}. The solid phase antibody was washed 3 times with the assay buffer and stored at 4°C. It was diluted for use in assay buffer to give 30% binding in the zero cortisol serum standard (B_0).

4.2.6.2 Assay procedure

Fifty microlitre aliquots of standards, quality controls and test samples were aliquoted in duplicate in to 12 x 55 mm tubes (Ciba Corning Diagnostics Ltd., Halstead, Essex, UK.). 100 µl of radiolabelled cortisol, diluted in assay buffer to give 30,000 to 40,000 counts per minute per tube and containing 1% (w/v) ANS and 200 µl of antibody, was added to all the tubes. The tubes were vortexed and incubated at room temperature for 1 hour. The "bound" and "unbound" cortisol

were separated by washing 3 times with 2.5 ml of 154 mM sodium chloride solution and after each wash centrifuging at 1500 g for 5 minutes. Finally the bound fraction was counted using a gamma-scintillation counter (Packard Auto-Gamma-3255, Pangbourne, Berks, UK). The results were subsequently analysed using the "SAS" system computer programme 632014 written by Dr. P. Edwards Department of Molecular Endocrinology, UCMSM, London, UK.

4.2.6.3 Cortisol assay validation for bovine samples

Accuracy, precision and the limit of detection for cortisol measurement in bovine sera were determined by the methods of Bangham (1993). Accuracy was measured from the recovery of known amounts of exogenous cortisol added (18.8, 32.5 and 95.7 nmol l^{-1}) to bovine serum. The mean ± SD % of recovery for cortisol was 108.6 ± 8.1 per cent (n = 18). The precision was determined as the coefficient of variation (CV) calculated for both intra- and interassay CVs in samples containing high, medium and low cortisol levels included in all assays. Intra-assay CV was < 9.9% (n = 12) for samples containing 16.2, 62.5 and 125.8 $nmol^{-1}$ and inter-assay CV was <18% (n = 18) for samples containing 12.5; 26.6 and 87.3 $nmol^{-1}$. The limit of detection of the assay was improved to permit accurate cortisol measurement and at 2 SD of maximum binding (B_o) the detection limit was 3.0 nmol l^{-1}.

4.2.7. Thyroxine (T_4)

The serum concentration of total T_4 was measured using a radioimmunoassay kit (Magic T_4) from Ciba Corning Diagnostic Limited (Halstead, Essex, UK). This is a competitive assay in which T_4 in standards, controls and samples compete with [^{125}I] labelled T_4 for a limited amount of T_4 antibody, which is covalently coupled to magnetic particles. Bound and unbound T_4 were separated by magnetic attraction followed by decantation of supernatant.

4.2.7.1 Thyroxine assay validation for bovine samples

The T_4 kit was designed for human plasma and needed validating for the bovine plasma prior to analysis. Validation was carried out by measuring the accuracy,

precision and the limit of detection as described for the cortisol assay (Section 4.2.6.3). In brief, T_4 was added as 30 µl volume to 1.0 ml volumes of serum (which covered the range of 32 - 98 nmol l^{-1}) and the recovery was 103.2 % ± 6.2 over 15 samples. The intra-assay coefficient of variation was < 6.7 % (n = 30) for samples containing 40.5, 64.2 and 75.4 nmol l^{-1} T_4 concentrations. The interassay CV's for 40.4, 64.8 and 92.3 nmol l^{-1} T_4 concentrations were at 7.8 %, 5.6 % and 8.7 % respectively over 5 assays. The limit of detection of the assay was 5 nmol l^{-1} being the T_4 concentration at 2 SD from the mean of the concentration with a zero serum sample from which T_4 had been removed by stripping with agarose coated charcoal.

4.2.8 Acute phase proteins

4.2.8.1 Haptoglobin

The haptoglobin (Hp) content in serum was measured by a modification of the method described by Conner *et al* (1988b) and based on previous report by Makimura and Suzuki (1982).

Reagents

Tetramethyl benzidine (TMB, Sigma Chemical Company) was used as the chromogen instead of the carcinogenic reagent *o*-dianisidine and the pH of the chromogen buffer (0.5g sodium EDTA; 15.6g Na H PO_4 .$2H_2O$ in 1 l distilled water) was adjusted to pH 3.8. A stock solution of TMB at 6 mg ml^{-1} was prepared in dimethyl sulphoxide and, for use in the assay, was diluted 100 x in chromogen buffer. A working haemoglobin (Hb) solution was freshly prepared by diluting a stock concentrated Hb solution to 0.3 mg ml^{-1} with saline for each assay. The Hb was quantified in the stock solution by a haematocrit analyser Roche ABX Minos Vet. (Roche products Ltd, Diagnostic Division, P.O. Box 8, Welwyn Garden City, Hertfordshire) The stock Hb solution was prepared according to Makimura and Suzuki (1982). Flat-bottomed Microtitre plates were obtained from Nunc, Gibco Ltd., (Paisley, UK.).

Standards

Serial dilutions (0 to 2.0 g l^{-1}) of acute phase bovine serum with a known amount of haptoglobin, quantified by comparison to purified Hp (Eckersall and Conner 1990), were used as standards. The dilutions were made in normal bovine serum and had a range from 0.03 to 2 gl^{-1} with the normal serum as zero standard.

Assay procedure

A 20 µl aliquot of acute phase serum was mixed with 100 µl of working Hb solution in a glass test tube (3 x 100 mm) and incubated for 10 minutes at room temperature. The reaction mixture was then diluted with 5 ml of saline, mixed and duplicate 20 µl aliquots delivered into the wells of a microtitre plate followed by 200 µl of chromogen buffer to each well and incubated for 1 hour at 37°C. At the end of the incubation period 50 µl of H_2O_2 substrate (12 µl of 30% H_2O_2 in 10 ml dH_2O) was added and incubated at 37°C until a strong blue colour developed in the maximum standard. Subsequently, 50 µl of 2M H_2SO_4 were added to all the wells to inhibit any further peroxidase activity. The plate was read using an ELISA plate reader at 450 nm and the haptoglobin concentration of the test sera calculated by comparison with the standards, using an immunoassay analysis computer programme (Titersoft, Flow Laboratories, UK). The standards were 2.0, 1.0, 0.5, 0.25, 0.13, 0.06, 0.03 g l^{-1}. The limit of sensitivity was 0.02 g l^{-1} and was calculated as 2 x SD of the zero standard. The intra-assay coefficients of variance were 6.0% and 5.0% respectively for thirty samples containing 0.5 and 1.0 Hp g l^{-1} concentrations. The interassay precision was determined by calculating the coefficients of variation obtained with samples containing 0.5 and 1.0 g l^{-1} of Hp and were 6.6% and 4.3%, respectively, over fifteen assays.

In previous reports results of this assay Hp concentrations have been expressed as mg haemoglobin binding capacity per decilitre (HbBC $100ml^{-1}$) which is based on the haemoglobin binding capacity of Hp. In this assay the Hp concentration was quantified with standards which had been standardised against

purified bovine Hp. To allow comparison with previous results a conversion factor of 29 should be used (Dr. P.D. Eckersall personal communication), thus 1 g l^{-1} = 29 mg HbBC 100ml^{-1}.

4.2.8.2 Serum Amyloid A

Serum amyloid A concentrations were determined by ELISA as described previously in Chapter 3. Plasma concentrations of SAA are expressed in $\mu g\ ml^{-1}$.

4.2.8.3 Ceruloplasmin

Ceruloplasmin (Cp) activity in the serum was estimated using the method of Sunderman and Nomoto (1970) using *p*-phenylenediamine dihydrochloride (PPD) from Sigma Chemical Company Ltd. (Poole, Dorset, UK.; P-1519) as substrate. The principle in this assay is that at pH 5.9 Cp catalyses the oxidation of PPD to yield a coloured compound which absorbs at a wavelength of 530 nm. The rate of formation of the coloured compound is proportional to the concentration of serum Cp. Correction was made for non-enzymatic oxidation of PPD by carrying out a blank assay in the presence of sodium azide which inhibits the oxidase activity of Cp. The difference between the two assays is proportional to the serum Cp concentration.

The pH optimum for the reaction of bovine ceruloplasmin was confirmed as pH 5.9 by testing 0.1M acetate buffers from pH 5 to 7.5. The activity of ceruloplasmin was expressed in oxidase units, where one oxidase unit is the amount of ceruloplasmin which alters the optical density at a rate of 0.01 unit per minute at 530 nm in a 1 cm light path under the conditions described (Conner *et al.* 1988b).

Reagents

p-phenylenediamine dihydrochloride and sodium azide (NaN_3) were from Sigma Chemicals (Poole, Dorset, UK). The assay was performed in 10 ml glass test tubes and a water bath at 37°C. Absorbance was measured on a spectrophotometer (Cecil, CE 373, Cambridge, UK).

Assay Buffer and Chromogen reagent

The assay buffer consisted of 430 ml of 0.2 M sodium acetate mixed with 70 ml of 0.2M acetic acid diluted to a litre with dH_2O. The chromogen reagent consisted of 27.8 mM PPD (0.5g PPD to 100 ml) in assay buffer which was adjusted to pH 5.9 with 1 M NaOH. This was prepared fresh as PPD is stable in solution for only 3 hours.

Assay Procedure

Aliquots of serum (100 µl) were added in quadruplicate to 3x100 mm glass tubes. Acetate buffer (2 ml) and 1 ml of buffered PPD was added to all tubes and 50 µl of 1.5M NaN_3 added immediately to 2 tubes to serve as sample blanks. All tubes were incubated for 30 minutes at 37°C, after which 50 µl of 1.5M NaN_3 were added to the untreated sample tubes. Absorbance were measured at 530 nm.

4.2.8.4 Fibrinogen

The concentration of plasma fibrinogen was determined by the Haematology laboratory of Department of Veterinary Pathology GUVS using a Diagen Fibrinogen Kit (Diagnostic Reagents Limited, Thames, Oxon). The assay principle was originally described by Clauss *et al.* (1957) and was based on the inverse relationship between the fibrinogen content of blood and the clotting time. However, blood samples were collected into EDTA which chelated calcium ions and the test had to be modified in consultation with the manufacturers, by adding 4 ml of 1M calcium chloride to 1 ml of thrombin reagent before the fibrinogen content was assayed.

4.2.9. Clinical Chemistry Profile

A biochemical profile focused on plasma protein and liver enzymes and metabolites was performed on plasma samples from Group I and control group using an automated biochemical analyser (Mira Roche Diagnostica, Welwyn Garden City, Herts, UK) with commercial assay kits (Roche Products Ltd., Herts, UK). The analytes included in the assessment of the responses in calves to

P. haemolytica infection were albumin, globulin, total protein, bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, gamma glutamyl transferase (γGT) and glutamate dehydrogenase (GLDH). However, only bilirubin and GLDH results are reported in the results section as these were the only two analytes to show a significant change following infection.

4.2.10 Post-mortem

Detailed post-mortem examination was restricted to the respiratory system and was performed by Dr. H.A. Gibbs of the Department of Veterinary Medicine. Lung lesions were examined grossly and pulmonary consolidation scores were mapped and finally photographed. Apart from one calf (Calf 9 in Group I) which died on the first night of the experiment, the calves were sacrificed by stunning with a captive bolt pistol followed by exsanguination. One calf (calf 3 in Group I) was slaughtered for humane reasons on day 1.

4.2.11 Statistical methods

Results are presented as group means ± SEM. Data obtained from infected and control calves comparing rectal temperature, respiratory rates, acute phase protein concentrations, hormone assays, haematology and the clinical chemistry profile were subjected to analysis of variance with repeated measures design using a computer statistical package ANIMAL DESIGNS 1, V 1.21 5/6 (Data International Service, Glasgow). Paired 't' tests were used to identify the time points at which particular group differences were significant. Iron, zinc and copper were analysed on the basis of their respective pre-infection concentrations. In experiments involving more than two treatment groups (Group I, Group II and control) comparisons were analysed by one way analysis of variance followed by a between group comparison test, the Newman-Keuls Multiple Range Test. Comparisons were considered significantly different at $P<0.05$.

4.3. RESULTS

Bovine pneumonic pasteurellosis was successfully established in both infected groups using the infection regime described above. All the infected calves showed signs of typical bovine pneumonic pasteurellosis by the afternoon of day 0 or by the morning of day 1 at the latest.

4.3.1. Clinical Signs

4.3.1.1 Demeanour

All calves remained bright and healthy during the acclimatisation period. Infected calves in Group I became dull, anorexic and pyrexic within six hours of inoculation (Appendix 4.0). Two calves died within two days of inoculation. Surviving animals remained dull and listless until they were sacrificed on day 6 post-inoculation. Group II calves were dull and anorexic during the early stages of infection but became brighter after day 3. Calves in the control group, inoculated with sterile PBS, remained bright and healthy throughout the experiment.

4.3.1.2 Rectal temperature

Individual rectal temperatures were recorded throughout the experimental period and are shown in Appendix 4.1. Mean (± SEM) daily rectal temperatures are presented graphically in Figure 4.1a.

Calves were afebrile during the acclimatisation period (rectal temperatures < 40°C). After the inoculation on day 0 there was a moderate rise in the mean rectal temperatures of both experimental groups. On the following day Group I calves showed other severe signs of BPP such as dullness and respiratory signs but showed no further rise in mean rectal temperature. The most likely reason for this was that one calf was sacrificed on humane grounds and the calf which died on day 1 of the experiment: these calves were moribund with falling rectal temperatures at the time of clinical examination (Appendix 4.1). From day 2 onwards the mean rectal temperature rose further and remained high for the rest of the experimental period. After day 0 the mean rectal temperature of Group II

calves was indistinguishable from the control calves and was similar to the pre-infection values.

4.3.1.3 Respiratory rate

Individual respiratory rates were recorded throughout experimental period and are presented in Appendix 4.2. Mean values for each of the groups between day -1 and day 5 are presented graphically in Figure 4.1b. No calves were noted to be tachypnoeic (which for this experiment was considered to be a respiratory rate equal to or greater than 60 min^{-1}) during the pre-infection period. There was a dramatic rise in group mean respiratory rates following infection on day 0 in all calves in Group I and II from means of between 20 and 30 min^{-1} to means in the region of 70 min^{-1} within 12 hours of infection. The respiratory rate of calves in the control group was raised slightly, (16 to 22 $\pm$ 3 min^{-1}) on day 1 and then decreased over the next 12 hours to the normal level of 18 min^{-1} for the remainder of the experiment.

4.3.1.4 Food intake

The group daily food intakes of the calves in Group I and II are shown in Figure 4.2a and in Appendix 4.3.1. The food intake of the calves infected with *P.haemolytica* A1 in Group I fell markedly from 1.3 kg per 50 kg live-weight during pre-infection period to zero on day 0 and remained severely depressed for the duration of the experiment. However, in Group II, food intake dropped by a third, from 1.5 to 1 kg per 50 mg body weight on day 0 but increased on day 3 with a slight reduction thereafter.

Individual live weights at various points over the experimental period are recorded in Appendix 4.3.2. Following infection on day 0 weight loss was seen in all infected calves but was most dramatic in the Group I calves which, as a group suffered a 9.9% drop in total body weight over the period day 0 to day 6 (Fig.4.2b). The total body weight of the calves in Group II remained unchanged (810 kg) throughout the experimental period .

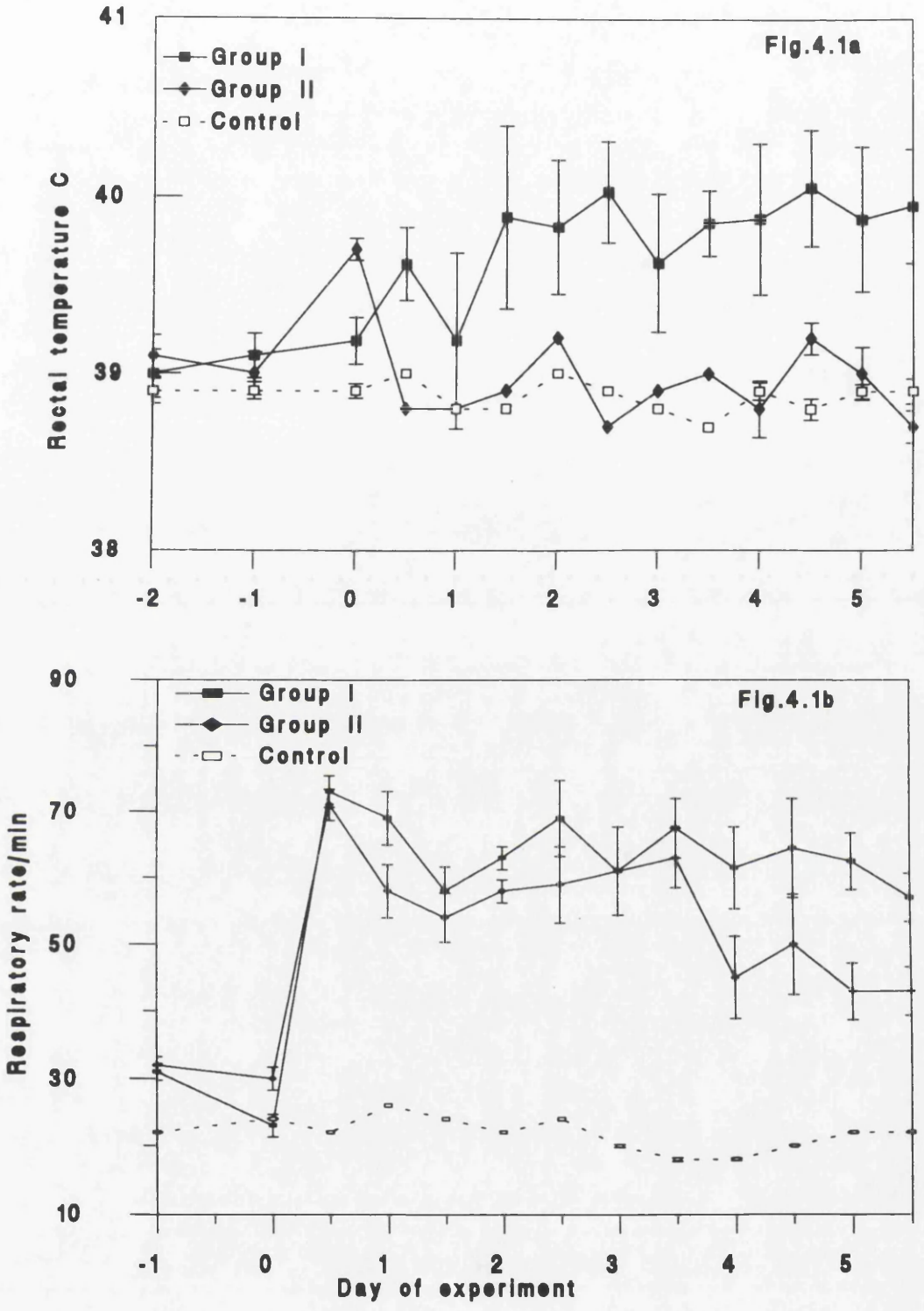
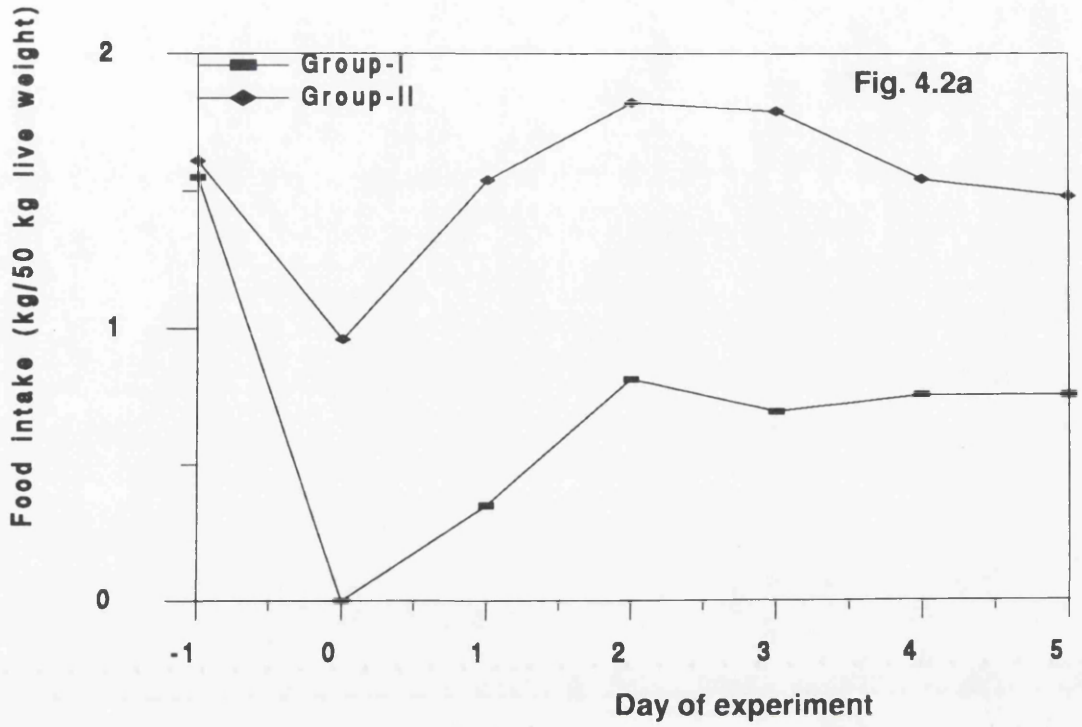


Figure 4.1 The mean (± SEM) rectal temperatures (Fig. 4.1a) and mean (± SEM) respiratory rates (Fig. 4.1b) of calves intra-tracheally inoculated with *P.haemolytica* A1. Calves in Group I (n=6) were inoculated with isolate SB/82/1 and the calves in Group II (n=6) were inoculated with isolate SC/82/1. The control group (n=5) received phosphate buffered saline intra-tracheally. Animals were examined daily at 10.00 hrs and 16.00 hrs. Two calves in Group I died of severe pasteurellosis on day 1.



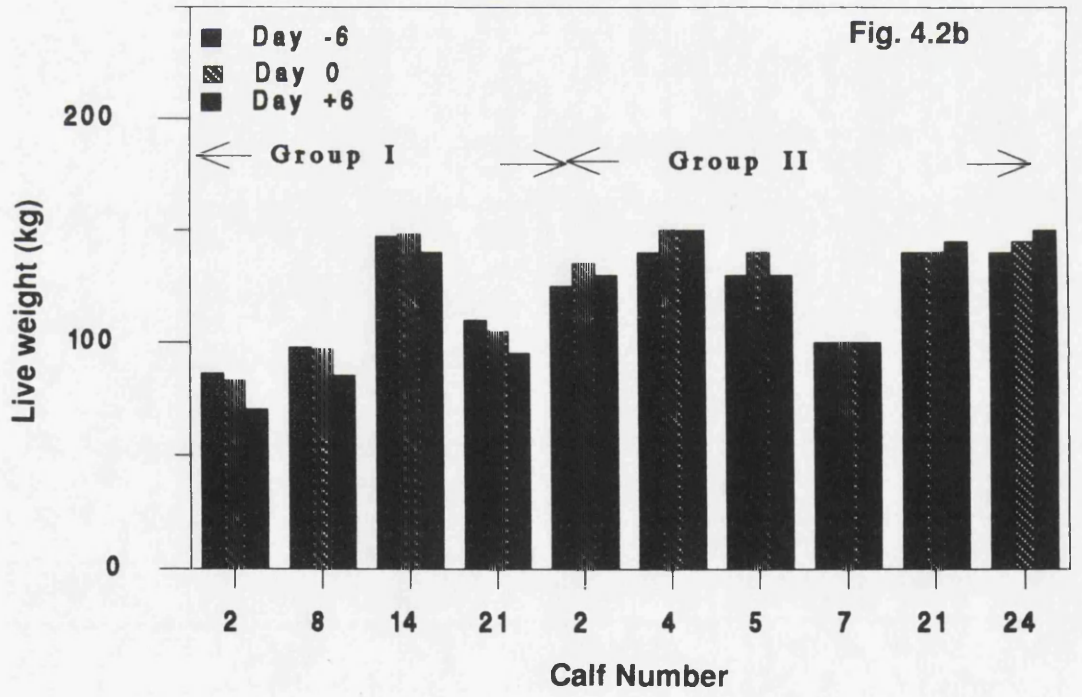


Figure 4.2 The daily total group food intake of calves in Group I (n=6) and Group II (n=6) (Fig. 4.2a). The individual live weights of calves in Group I (n=4) and Group II (n=6) 6 days before infection, on the day of infection and 6 days after the infection (Fig. 4.2b). Two calves in Group I died of severe pasteurellosis on day 1.

4.3.2. Haematology

Though several haematological parameters were measured (4.2.4.) only the parameters that were affected by *P. haemolytica* infection are presented. The daily mean ± SEM for total leukocyte count, neutrophils, monocytes and lymphocytes (Group II) are presented graphically in Figure 4.3a. The individual WBC counts and the neutrophil count are presented in Appendix 4.4.

In the control calves no change was observed in total leukocyte count, neutrophils, monocytes or lymphocytes, after the PBS inoculation. In the calves of Group II, a significant increase ($P< 0.05$) was found in mean total leukocyte counts by 4 hours post-inoculation. This increase was reflected by a significant rise ($P< 0.05$) in neutrophil counts which included numerous band neutrophils (Fig. 4.3a). The total leukocyte and neutrophil counts reached a peak on day 1 and then gradually dropped over days 2 to 3. By day 4 the count was equivalent to pre-infection levels. Differences between Group II and control calves in lymphocyte or monocyte counts were not statistically significant, although at 4 hours post-inoculation the lymphocyte values did drop slightly below pre-infection level. The Group II mean (± SEM) for RBC counts and haematocrit are given in Figures 4.3b and 4.3c respectively. Both RBC and haematocrit showed a significant ($P<0.05$) drop from the time of infection to day 5 post-inoculation.

4.3.3. Trace Minerals

The individual daily serum Fe, Zn and Cu concentrations of the infected Group I are given in Appendix 4.5 and the daily group mean (± SEM) for Fe, Zn and Cu concentrations of infected and the control group are presented graphically in Figures 4.4a and 4.4b, respectively. The pre-infection concentrations of the trace minerals were significantly high in the infected group compared to the control group which was purchased from a different farm. The

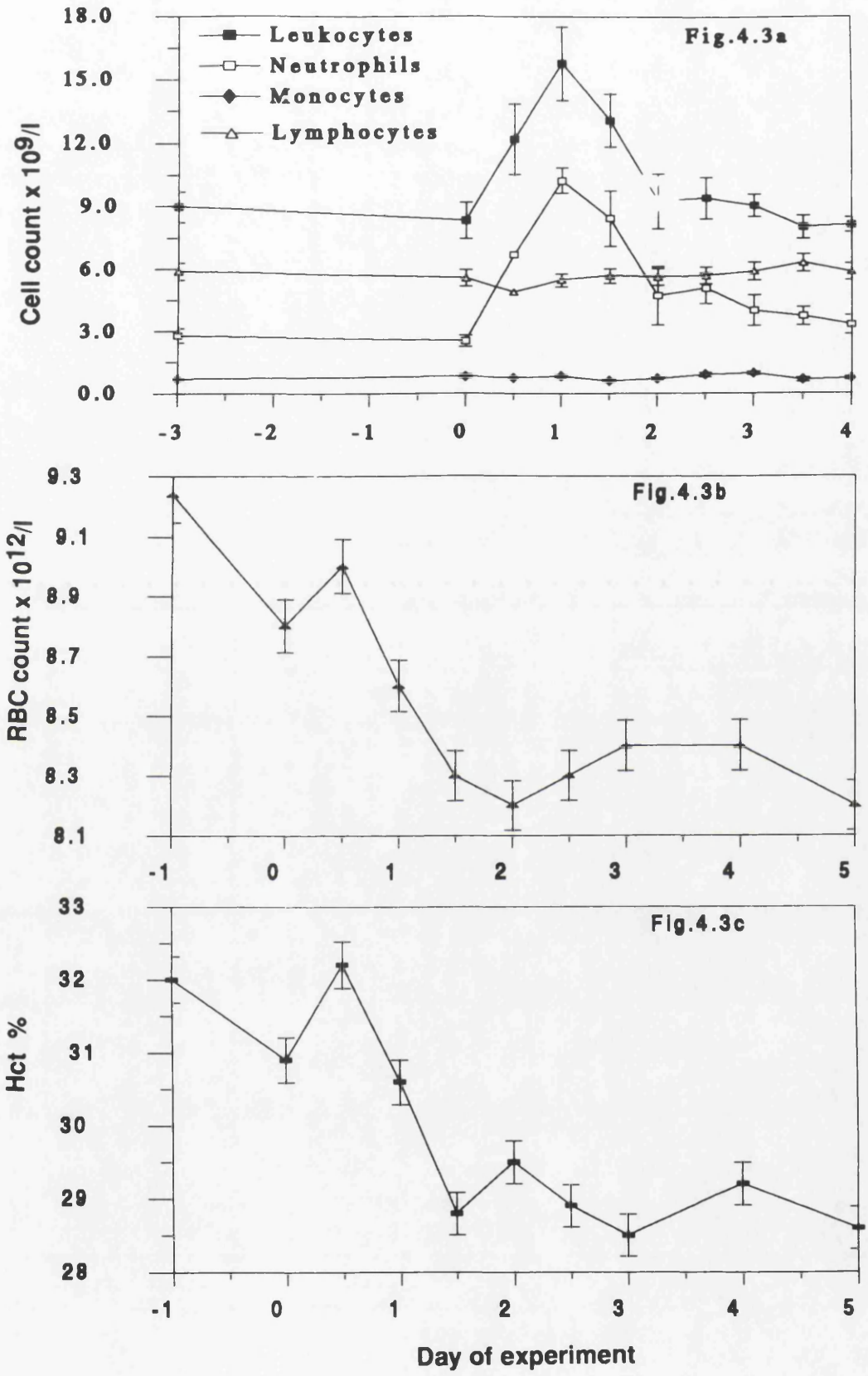


Figure 4.3 The mean (± SEM) total leukocyte, neutrophil, monocyte and lymphocyte counts (Fig. 4.3a), the total red blood cell counts (Fig. 4.3b) and haematocrit (Fig. 4.3c) of calves experimentally infected with P. *haemolytica* A1 isolate SC/82/1.

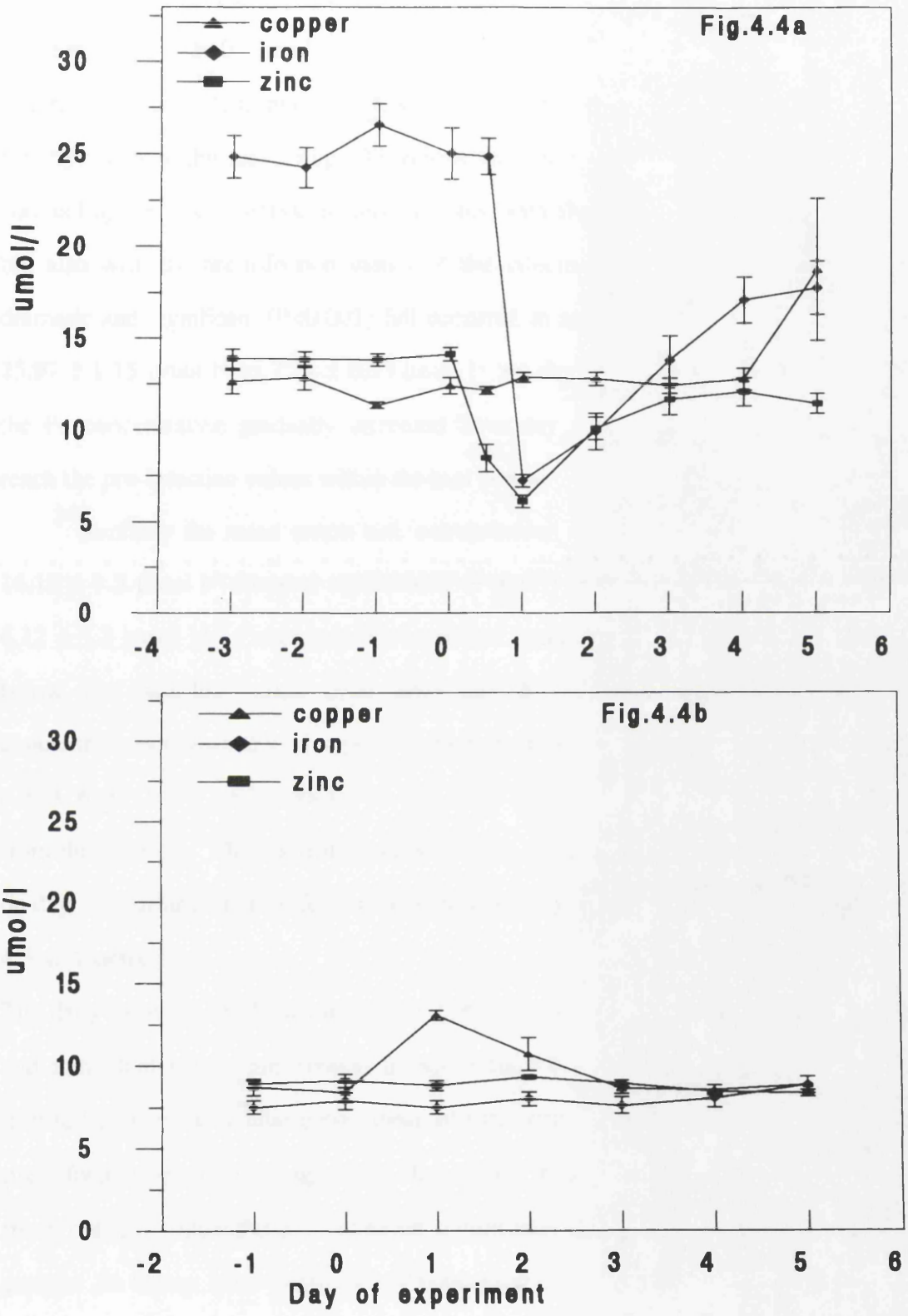


Figure 4.4 Mean (± SEM) serum iron, zinc and copper concentrations of calves (n=6) infected intra-tracheally with *P. haemolytica* (Fig 4.4a). The control calves (n=5) received phosphate buffered saline intra-tracheally(4.4b). Two calves in the infected group died on day 1 with severe pasteurellosis.

values in the control group were found to be at the lower end of the reference range (Fe: 10.2 - 29.0 μmol l^{-1}, Zn: 6.0 - 16.0 μmol l^{-1} and Cu: 8.0 - 11 μmol l^{-1}; Reference values, Department of Animal Husbandry, GUVS; Kaneko 1989) for healthy calves of this age group. Therefore, statistical analysis was carried out by comparing the post-infection results not only with the control group (ANOVA) but also with the pre-infection values of the infected group (paired t-test). A dramatic and significant ($P<0.001$) fall occurred in serum Fe concentration from 25.97 ± 1.15 μmol l^{-1} to 7.24 ± 0.33 μmol l^{-1} on day 1 post-infection. Although the Fe concentration gradually increased from day 2 the concentration did not reach the pre-infection values within the trial period.

Similarly the mean serum zinc concentration which, before infection, was 14.13 ± 1.5 μmol l^{-1} dropped significantly ($P<0.05$) on day 1 post-inoculation to 6.12 ± 1.3 μmol l^{-1}. Concentrations increased gradually thereafter but remained below the base-line value even after day 5. The mean serum copper concentrations remained unchanged (12.39 ± 0.4 μmol l^{-1}) until day 4 and showed a 50% rise (18.7 ± 3.87 μmol l^{-1}) on day 5 but this was not significantly different from the controls. The control group showed a slight rise in mean serum copper on day 1, returning to pre-infection concentrations by day 3.

4.3.4. Cortisol

The daily mean (± SEM) serum cortisol concentrations are shown in Figure 4.5a. and individual results are present in Appendix 4.6. The control and both the infected groups had similar group mean plasma cortisol concentrations during the pre-infection period in a range of 3 - 30 nmol l^{-1}. Following inoculation the group mean cortisol concentrations increased significantly ($P < 0.001$) in both infected groups. In Group I the mean cortisol concentration continued to rise throughout the experimental period, whereas in Group II the mean cortisol concentration rose from the pre-infection level of 16 ± 1.6 nmol l^{-1} to reach 43 ± 2.7 nmol l^{-1} on day 1 then showed a gradually decline after day 3.

4.3.5. Thyroxine

Daily variation in mean (± SEM) serum T_4 concentration in the control and the infected groups are shown in Figure 4.5b and the individual results are presented in Appendix 4.7. On day 1 post-infection, T_4 concentrations were significantly reduced in both infected groups. In Group I the mean T_4 concentration decreased from 65 (± 7.5) to 17.9 (± 12.1) nmol l^{-1} while in Group II the drop was from 60 (± 4) nmol $l^{-1}$to 36 (± 7) nmol l^{-1}. The two calves that died in Group I had low concentrations of T_4 on day 1 of <5 nmol l^{-1} which was the detection limit of the assay. The serum T_4 concentration of the control group ranged between 53.6-43.4 nmol l^{-1}. There was no change in the T_4 serum concentration after PBS administration to the control group.

4.3.6. Acute phase proteins

The individual daily values for Hp, serum amyloid A, fibrinogen (Fb) and ceruloplasmin concentrations are shown in Appendices 4. 8 to 4.11. Hp and SAA were undetectable in pre-infection samples while the mean Fb for the group II was normal (250 mg ml^{-1}). Within 24 hours of infection, serum levels of Hp, SAA and Fb significantly increased, while ceruloplasmin remained unchanged until day 4 post-infection. The main increases occurred in Hp, SAA and Fb concentrations during day 1 and 2. After the peak of the serum concentration on day 2 the concentration of SAA and Hp declined rapidly but during the trial period concentrations did not declined to the pre-infection levels. The plasma Fb was maintained at a raised concentration for 4 days till the end of the experiment in Group II.

4.3.6.1 Haptoglobin

Haptoglobin concentrations were, 0.6 g l^{-1} in all groups during the pre-infection period but increased significantly ($P < 0.001$) on day 1 and reached a peak value of 2.0 to 2.5 g l^{-1} by day 2 (Fig. 4.6a.). Thereafter, values declined gradually. The concentration of haptoglobin was significantly higher in both infected groups

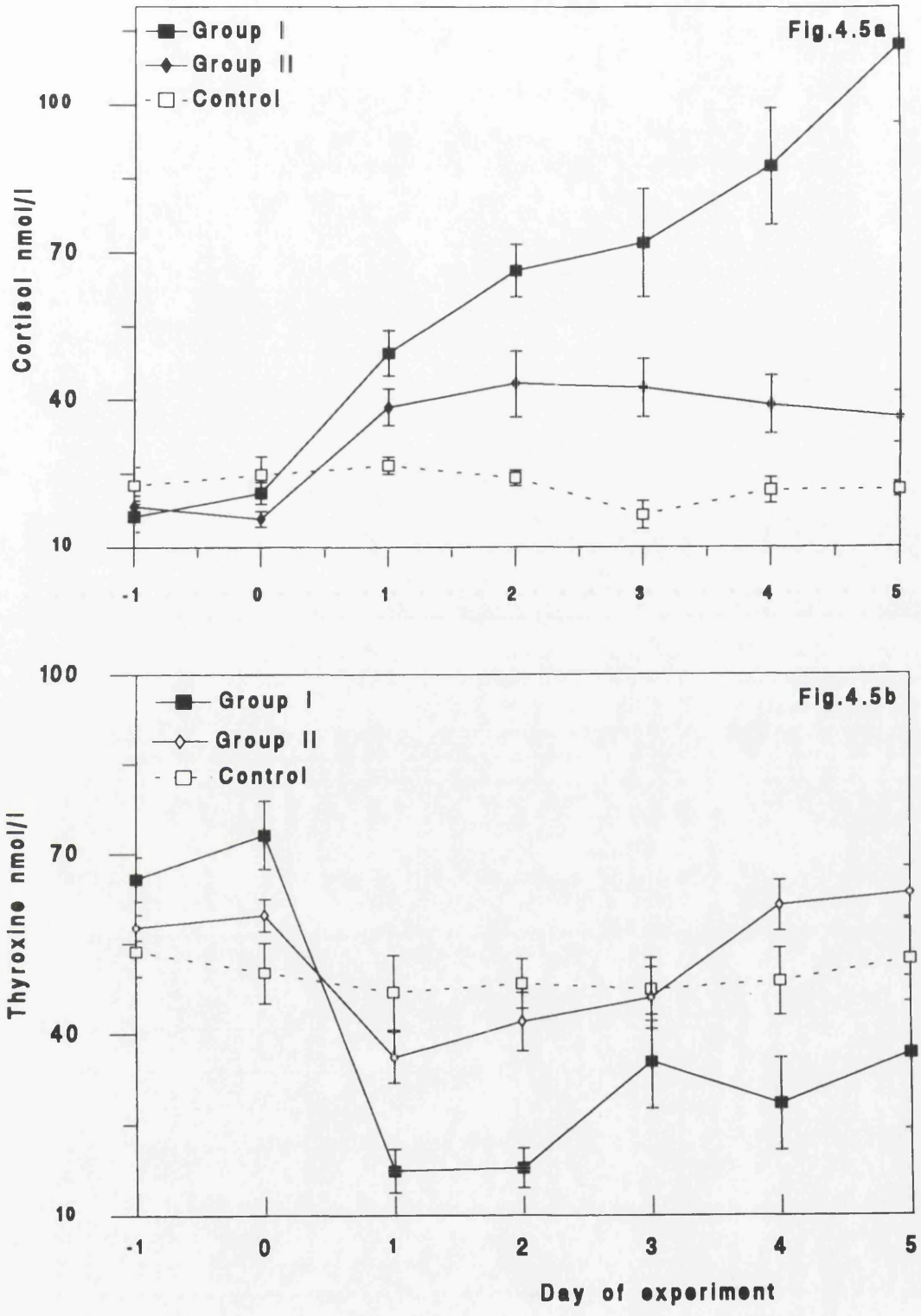


Figure 4.5 Mean (± SEM) plasma cortisol (Fig. 4.5a) and thyroxine concentrations (Fig. 4.5b) of calves infected intra-tracheally with *P haemolytica* A1 in Group I (isolate SB/82/1) and Group II (isolate SC/82/1). The control group received phosphate buffered saline intra-tracheally. Two calves in Group I died on day 1 with severe pasteurellosis.

when compared to the controls ($P < 0.001$) and there was no significant difference between the two infected groups in Hp concentration.

4.3.6.2 Serum amyloid A

The SAA concentration in both Group I and Group II was close to the detection limit on day 0 (<3 $\mu g\ ml^{-1}$) but was slightly higher in the control group. On day 1 the SAA concentration increased in both infected groups while the control calves remained unchanged (Fig. 4.6b). In Group I the concentration of SAA was significantly higher than Group II ($P < 0.05$) and controls ($P < 0.001$). Peak concentration occurred on day 2 with a mean (± SEM) 133.5 ± 12 $\mu g\ ml^{-1}$ SAA in Group I and 73.0 ± 6 $\mu g\ ml^{-1}$ SAA in Group II. The SAA concentration declined rapidly from day 3 but during the experimental period the levels remained higher than the controls.

4.3.6.3 Fibrinogen

Fibrinogen concentration was measured only in the Group II calves and the controls, and the daily group means (± SEM) are shown in Fig. 4.7a. The mean pre-infection plasma concentration for Fb was 200 ± 5 mg dl^{-1} and the concentration rose 4 hours after infection, reaching a peak of 800 mg dl^{-1}. On day 2 the Fb concentration was 1200 ± 6 mg dl^{-1} and this increase was found to be highly significant ($P < 0.001$). Concentration fell between day 2 and day 3 to 800 mg dl^{-1} but then remained at this level till the end of the experiment.

4.3.6.4 Ceruloplasmin

The daily group mean (± SEM) of serum ceruloplasmin in Group I and Group II are shown in Fig. 4.7b. From a mean pre-infection concentration of 0.1 (± 0.04) oxidase units, ceruloplasmin showed little variation in either group, although a slight rise to 0.22 (± 0.04) oxidase units was observed on the day 5 in group I. However, this increase was not significant ($P > 0.05$).

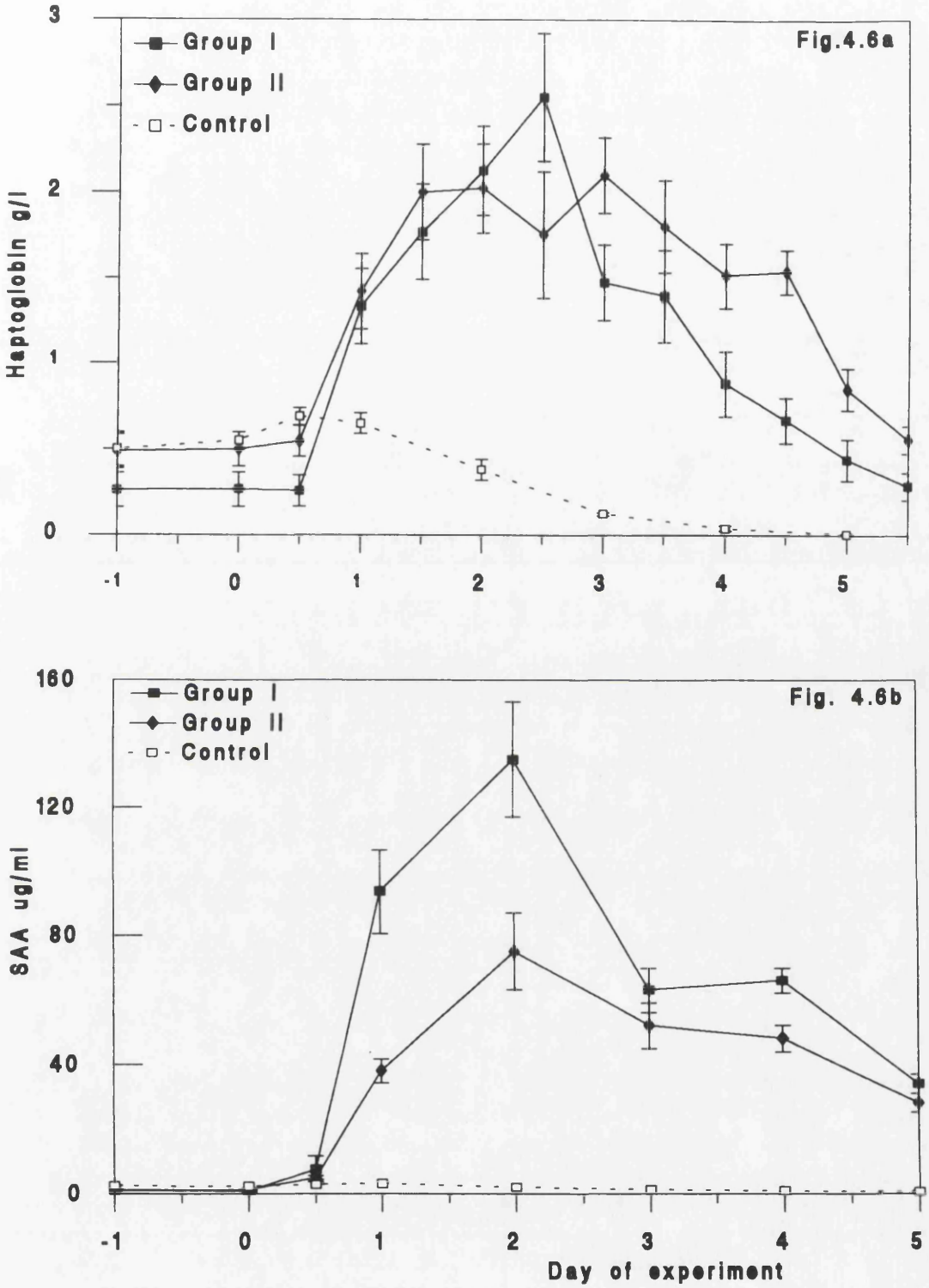


Figure 4.6 Mean (± SEM) haptoglobin (Fig. 4.6a) and serum amyloid A (Fig. 4.6b) concentrations of calves infected intra-tracheally with *P. haemolytica* A1 in Group I (isolate SB/82/1) and Group II (isolate SC/82/1). The control group received phosphate buffered saline intra-tracheally. Two calves in Group I died on day 1 with severe pasteurellosis.

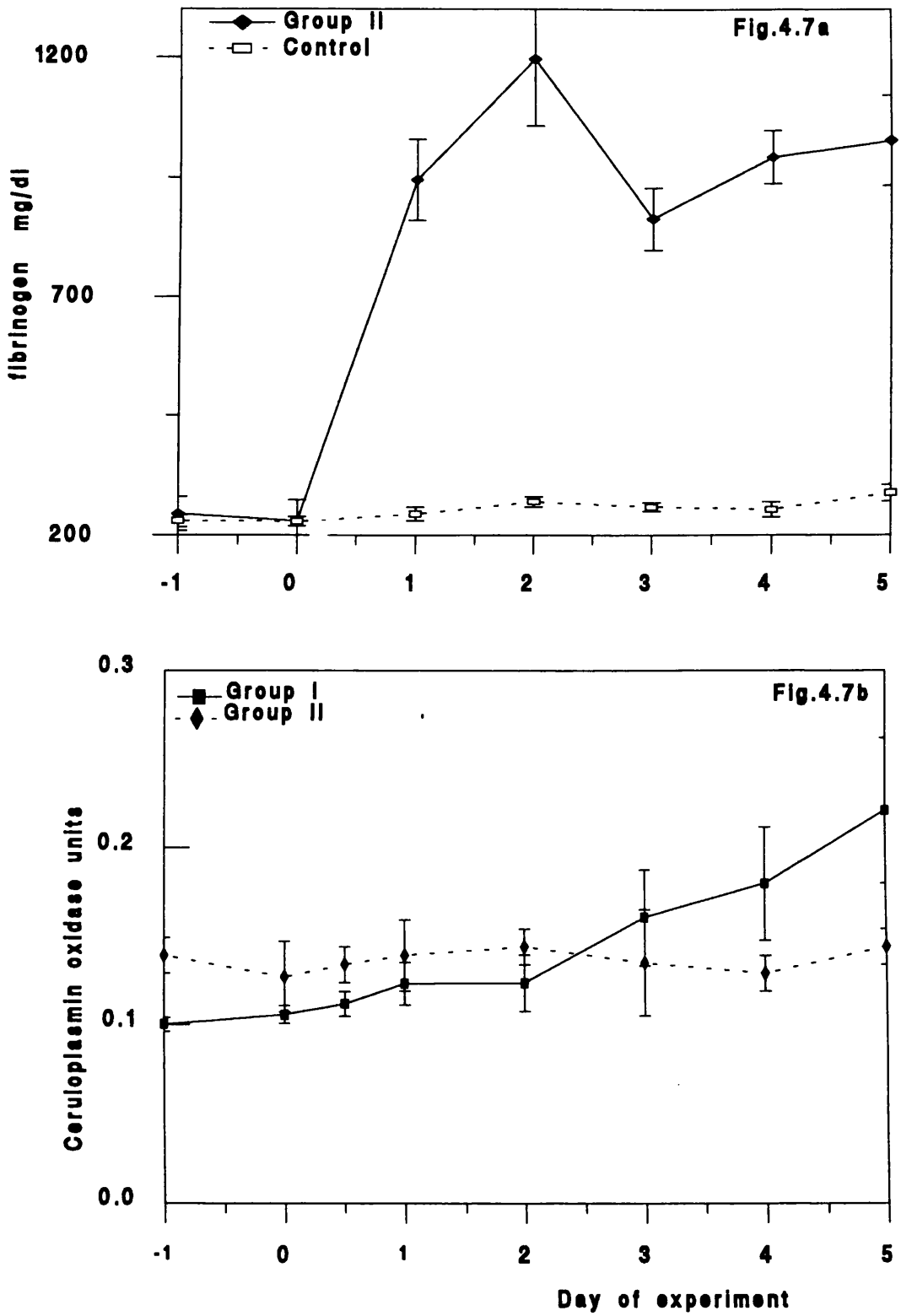


Figure 4.7 Mean (± SEM) fibrinogen (Fig. 4.8a) and ceruloplasmin (Fig. 4.8b) concentrations of calves infected intra-tracheally with *P. haemolytica* A1 in Group I (isolate SB/82/1) and Group II (isolate SC/82/1). The control group received phosphate buffered saline intra-tracheally. Two calves in Group I died on day 1 with severe pasteurellosis.

4.3.7. Clinical Chemistry Profile

Most analytes estimated by routine clinical chemistry were not affected by *P. haemolytica* infection.

4.3.7.1 Glutamate dehydrogenase activity

The Group I daily mean (± SEM) plasma GLDH activity are given in Figure 4.8a. In Group I there was a significant reduction ($P < 0.05$) in the GLDH activity, reducing from 43.38 U l^{-1} on day 0 to 13.12 U l^{-1} on day 4. Thereafter, levels increased gradually but within the trial period the pre-infection levels were not reached. The GLDH activity was unaltered in the control group.

4.3.7.2 Bilirubin

The pre-infection mean (± SEM) plasma concentration of bilirubin in Group I calves was 2.0 mg ml^{-1} and increased to a maximal value of 10.6 (± 0.2) mg ml^{-1} by day 2 post-infection and, despite the subsequent reduction, remained significantly ($P<0.05$) greater in infected calves than in the controls calves during the experimental period (Fig. 4.8b).

4.3.8. Post-mortem report

Group I

The pulmonary consolidation scores for the calves that were killed on humane grounds or died on first and the second day of the experiment was 52.7% and for the calves which were killed on the day 6 of the experiment was 61.8%. The group mean pulmonary score was 58.7% with a range of 40.3% to 87.8%. (Appendix 4.12). Pulmonary consolidation maps of individual calves are presented in Figures 4.9a to 4.9d. Lesions characteristic of an acute exudative fibrinous pneumonia and considered typical of those associated with bovine pneumonic pasteurellosis due to infection with *P. haemolytica* A1 infection were found in the calves of Group I. These lesions varied from widespread areas with a severe exudative fibrinous pneumonia with consolidation and pleurisy (Figures 4.10a. and 4.10b) to mild acute pneumonic lung lesions is present (Figure 4.10c).

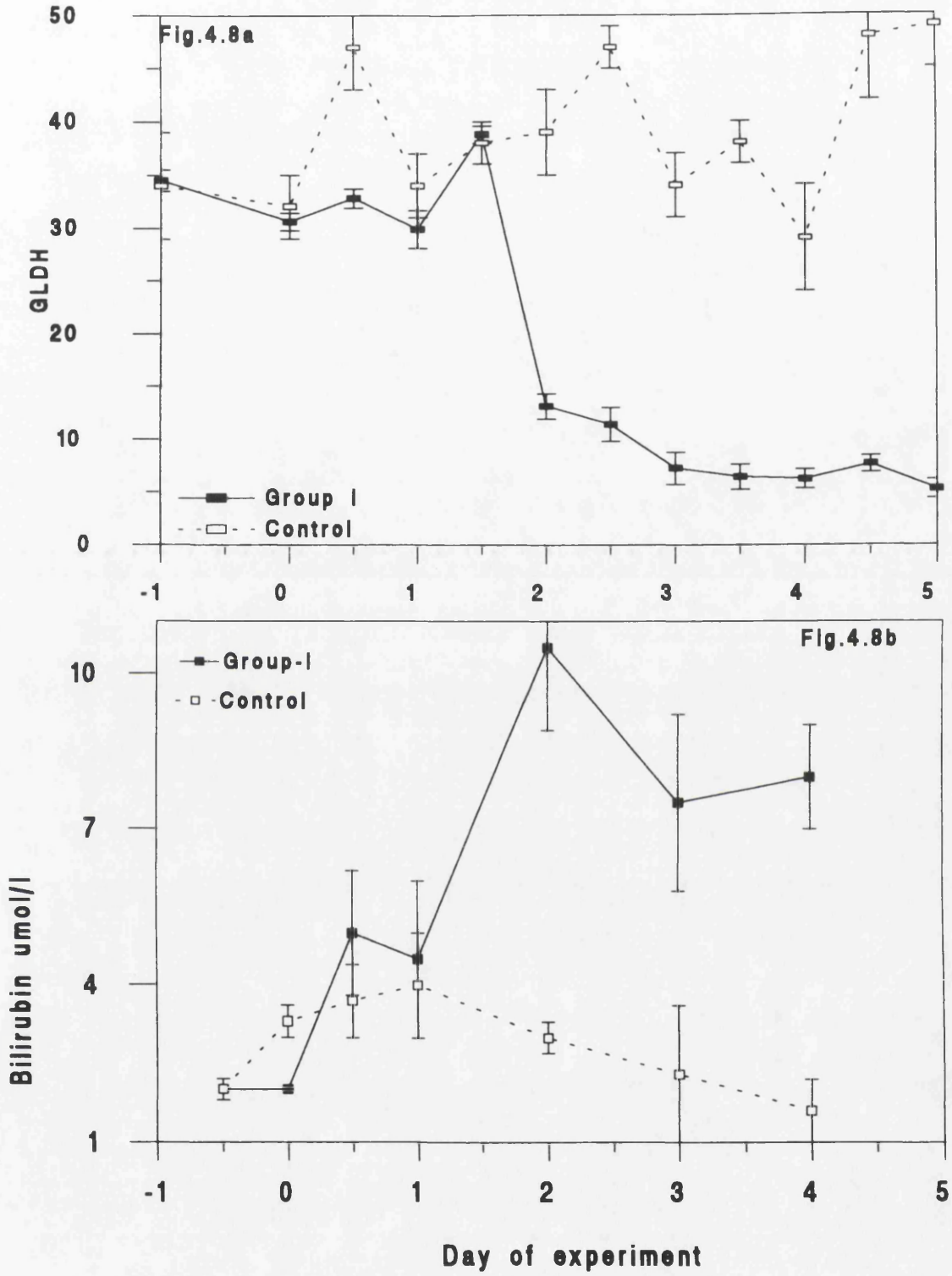


Figure 4.8. The mean (± SEM) glutamate dehydrogenase activities (Fig. 4.8a) and bilirubin concentrations (Fig 4.8b) of calves infected intra-tracheally with *P. haemolytica* A1 in Group I (isolate SB/82/1) and the control group. The control group received phosphate buffered saline intra-tracheally. Two calves in Group I died on day 1 with severe pasteurellosis.

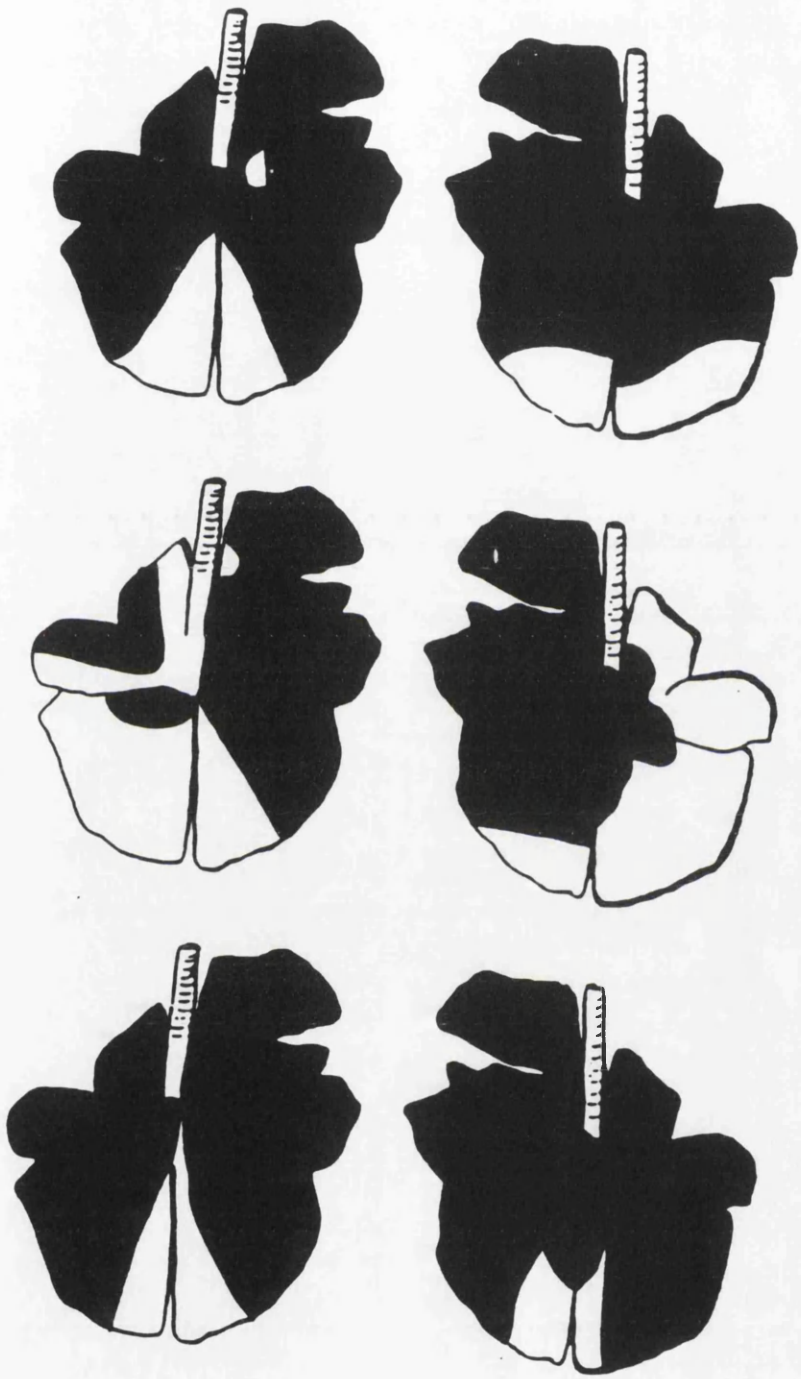


Figure 4.9a Pulmonary score charts of calf 2, 3 and 8 infected intra-tracheally with *P. haemolytica* A1 isolate SB/82/1 in Group I.

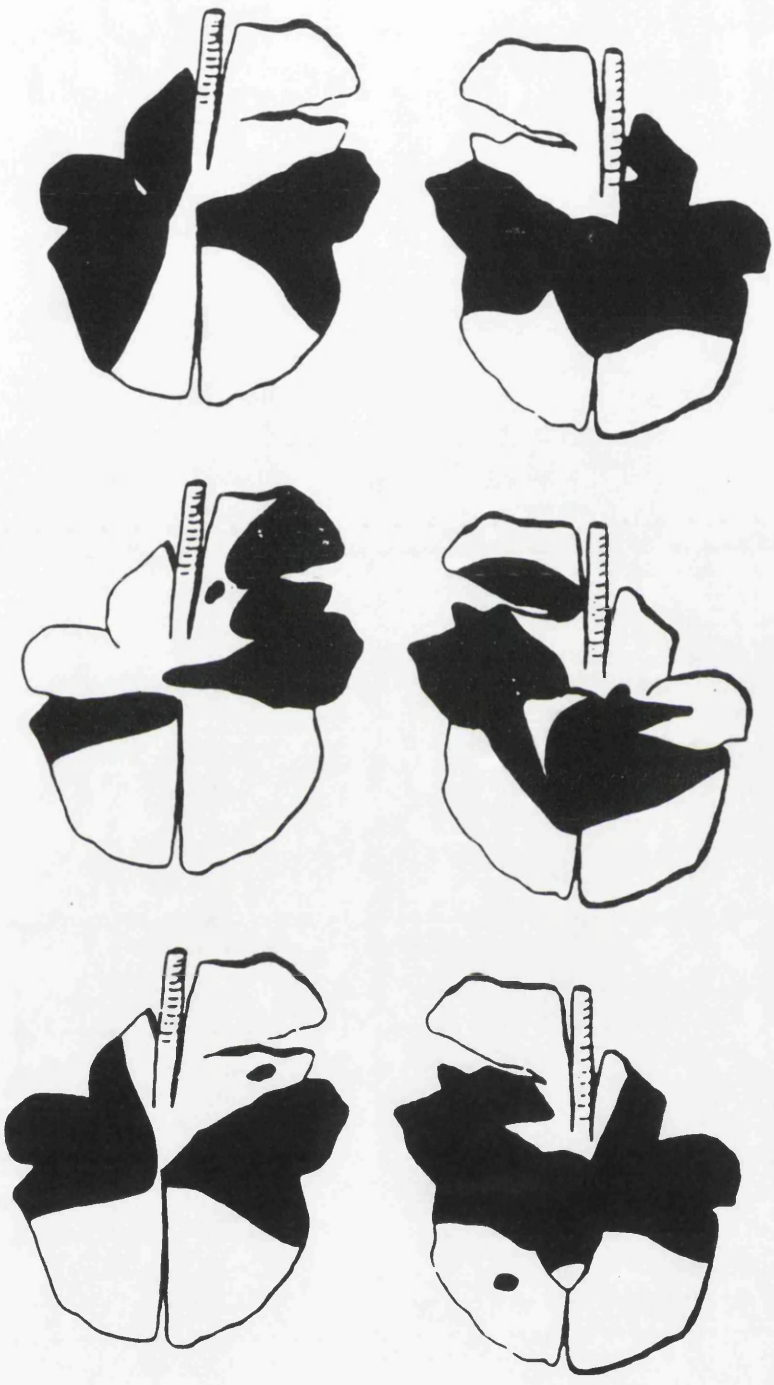


Figure 4.9b Pulmonary score charts of calf 9, 14 and 21 infected intra-tracheally with *P. haemolytica* A1 isolate SB/82/1 in Group I.

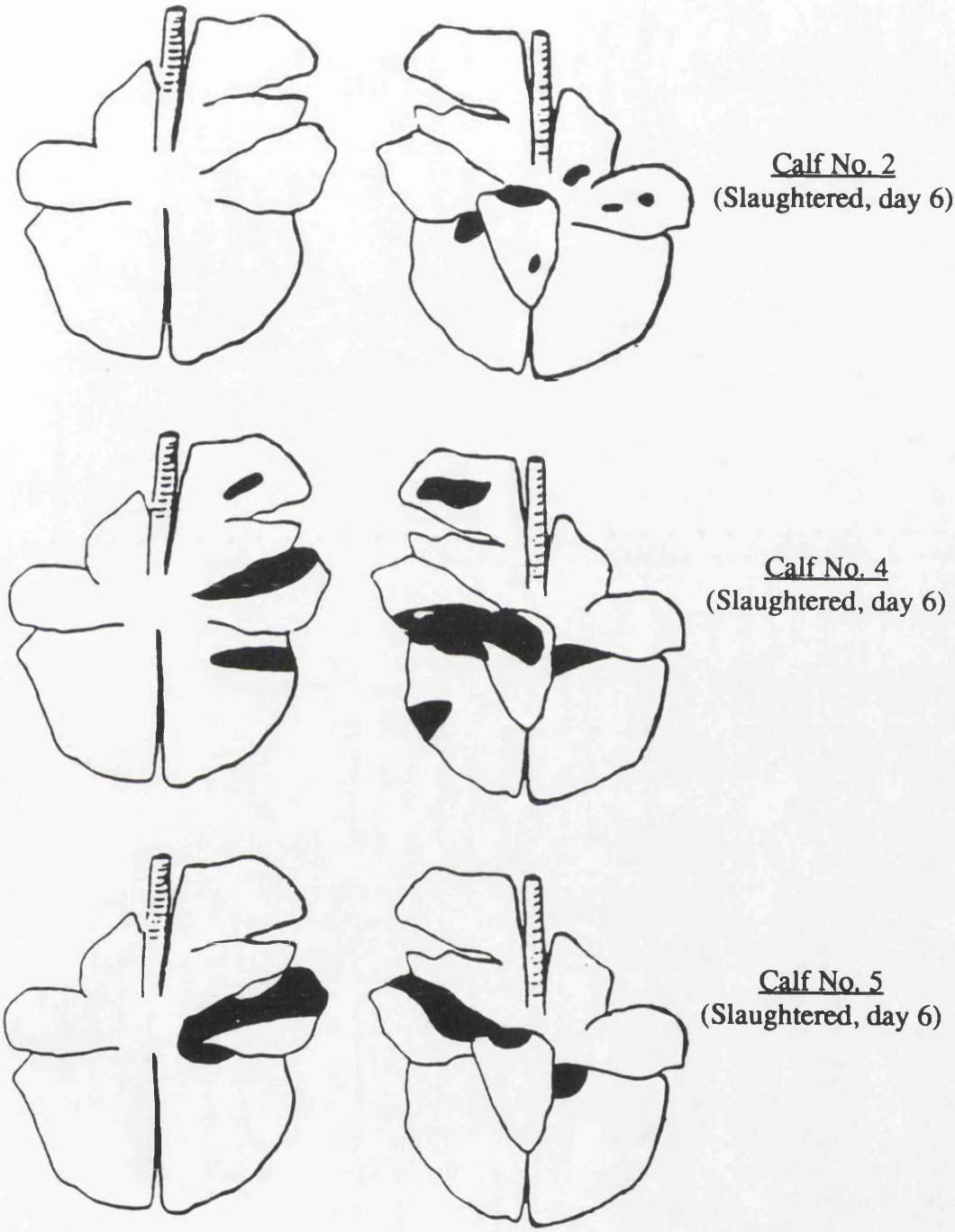
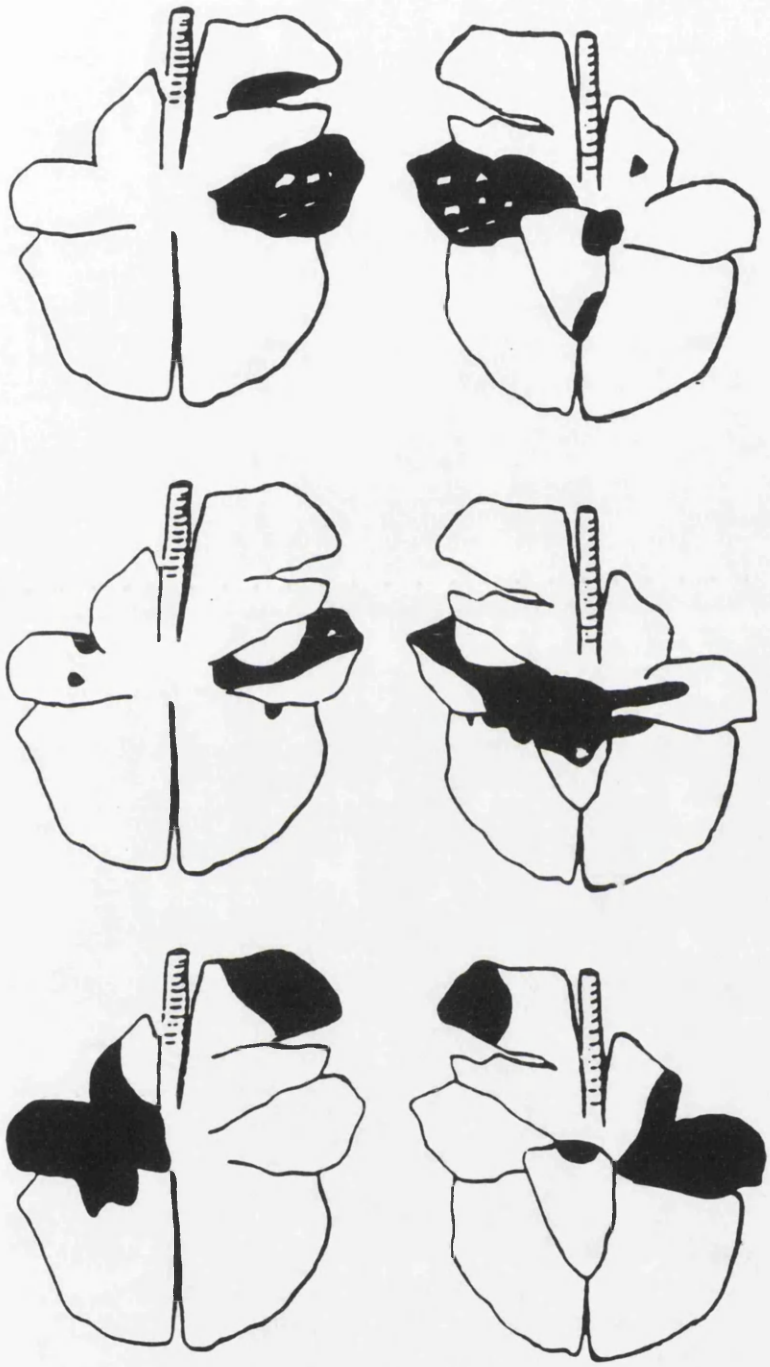


Figure 4.9c Pulmonary score charts of calf 2, 4 and 5 infected intra-tracheally with *P. haemolytica* A1 isolate SC/82/1 in Group II.



Calf No. 7
(Slaughtered, day 6)

Calf No. 21
(Slaughtered, day 6)

Calf No. 24
(Slaughtered, day 6)

Figure 4.9d Pulmonary score charts of calf 7, 21 and 24 infected intra-tracheally with *P. haemolytica* A1 isolate SC/82/1 in Group II.

Figure 4.10a Dorsal view of the lungs of calf 2 (Group 1) showing severe acute fibrinous pneumonia with pleurisy. The animal was infected with *P. haemolytica* A1 isolate SB/82/1.

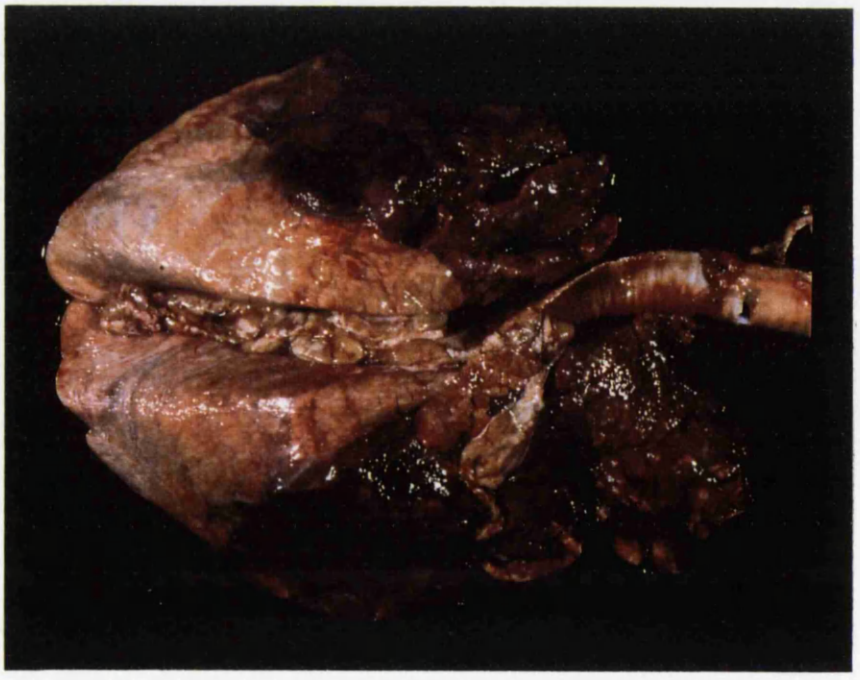


Figure 4.10b Dorsal view of lungs of calf 8 (Group I) showing severe acute fibrinous pneumonia. The animal was infected with *P. haemolytica* A1 isolate SC/82/1.

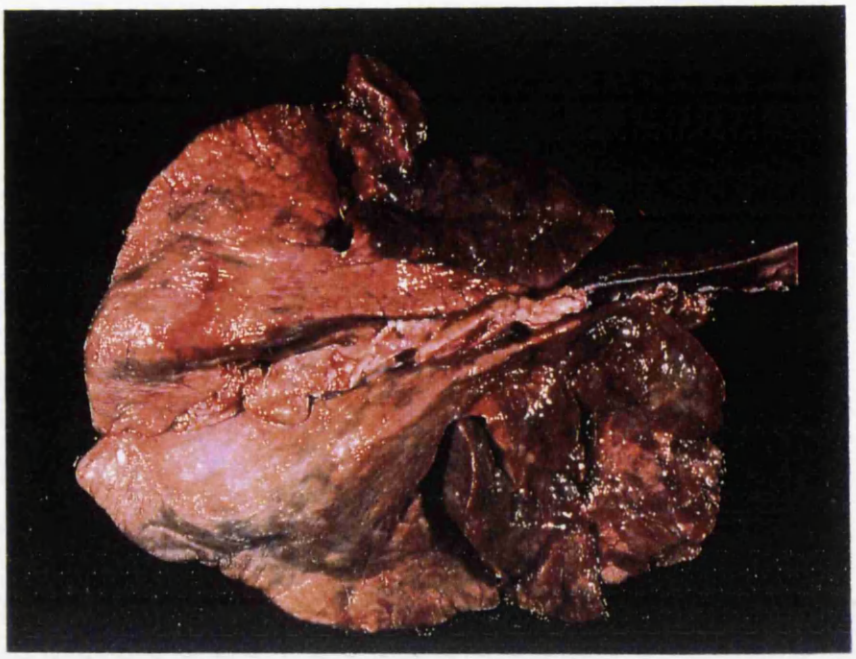


Figure 4.10c Dorsal view of lungs of calf 5 (Group II) showing mildly acute pneumonic lung lesions. The animal was inoculated with *P. haemolytica* A1 isolate SC/82/1.

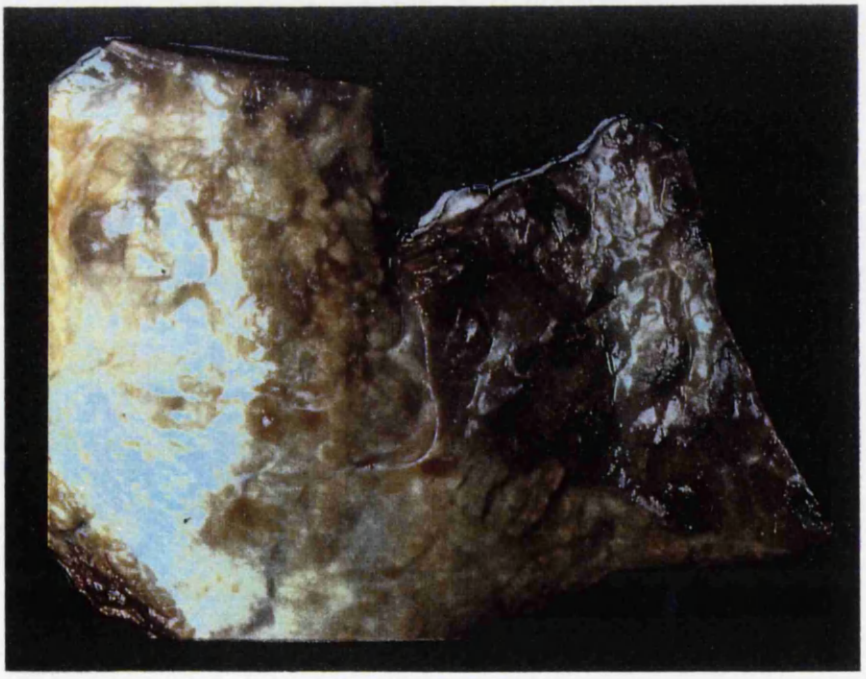


Figure 4.10d A part of a lung from an infected animal (calf 14; Group I) showing a typical nodule (arrow) produced by *P. haemolytica* A 1.

Some animals had resolving nodular lesions where small foci of fibrinous pneumonia became encapsulated (4.10d).

Group II

The group mean pulmonary consolidation scores was 10.3% with a range from 1.15% to 19.54% (Appendix 4.12). All calves of Group II had areas of acute exudative fibrinous pneumonia with fibrinous pleurisy overlying the anterior lung lobes (Figure 4.10a) but these lesions were smaller and more discrete than those seen in Group I. A few calves in Group II had very low-grade lesions of cuffing pneumonia.

Control

At necropsy, none of the calves in the control group had pulmonary lesions that represent BPP.

4.4. Discussion

The two objectives of these studies described in this chapter were to extend our knowledge of the acute phase reaction in cattle to infection with *P.haemolytica* A1 and to determined whether the response varied when induced by different field isolates of this Gram-negative bacterium. The results pertaining to the former objectives are derived largely from the experimental infection with isolate SB/82/1 (Group I) apart from the assessment of the haematological aspects of the systemic host response which were investigated with isolate SC/82/1 (Group II). This discussion will first cover all these new finding on the APR in bovine pasteurellosis and will subsequently compare those acute phase reactants which were monitored in Group 1 and Group II to consider whether different field isolates have a similar or differing effects on the APR.

The post-mortem reports, the results for rectal temperature, respiratory rate, demeanour and post-mortem findings confirmed that calves inoculated with isolate SB/82/1 (Group I) developed a severe infection typical of BPP. In contrast

calves inoculated with isolate SC/82/1 (Group II) had a less severe infection indicated by rectal temperature and post-mortem findings. Fever and a rise in respiratory rate are well established signs of BPP in the field (Hepburn 1925) and in experimental models of the disease (Gibbs *et al.* 1984; Dalgleish 1989). These clinical signs of the disease are likely to be caused by cytokine release (Whiteley 1992; Pace *et al.* 1993).

Gram-negative bacteria release several toxins such as endotoxins, leukotoxins and fimbrial antigens which trigger macrophages and monocytes to stimulate the release of cytokines such as IL-1 IL-6 and TNF_{α} (Dinarello *et al.* 1989; Atkins 1989). Several studies in laboratory animals have shown that the development of fever is a result of LPS or its mediators IL-1, IL-6 and TNF_{α}, the pyrogenic cytokines, which in turn induce the anterior hypothalamus to release prostaglandin E_1 and bring about pyrogenesis (Dinarello 1984a) by increasing the thermo-regulatory set point (Kluger 1989).

Fever is one of the characteristic features of the systemic APR. Moderate fever may improve the host's defence in several ways, such as increasing migration of leukocytes to the site of infection and by enhancing their phagocytic and bactericidal activities. These aspects of inflammation and the immune response do not function optimally at normal body temperature (Kluger 1975; van Merit *et al.* 1992).

In previous studies of the APR in cattle infected with *P.haemolytica* A1, the distinctive rapid increase in APP such as Hp, α_1 acid glycoprotein and α_1 antitrypsin has been described (Conner *et al.* 1989). This investigation has extended our knowledge by demonstrating that SAA is a major bovine APP and for the first time, by revealing simultaneous alterations in the other classes of acute phase reactants, notably the trace elements, hormones and neutrophil count in calves with BPP.

The control group was brought to the GUVS at a different time to minimise the risk of cross-infection during handling and sampling and were not purchased from the same farm as the infected groups with the result that the control and infected group of calves had different basal iron, zinc and copper values, due to the difference in the nutrition and the management practices of the two farms. In order to avoid this discrepancy the mineral changes observed in the infected group were compared with the pre-infection values as well as those obtained with the control groups.

The immediate drop, following infection, in circulating concentrations of the trace elements Fe and Zn found in calves has been shown to occur in acute infection in species such as sheep where trauma, digestive disorders and stress can reduce the circulating zinc and copper (Corrigal *et al.* 1976; Pfeffer and Rogers 1989). In goats, *E coli* endotoxin administration caused a reduction in serum iron and zinc concentrations (van Merit *et al.* 1984). Hypoferraemia during bacterial infection has also been studied extensively in laboratory animals after treatment with bacterial components, particularly LPS (Kampschmidt and Upchurch 1969; Butler *et al.* 1973; Turchik and Bornstein 1980). Interestingly an injection of iron or zinc reduced the survival rate of experimentally infected animals (Kluger and Rothenburgh 1979). From these and other studies the postulate has arisen that the benefit to the host of a reduction in trace elements is to limit the available supply of iron and zinc which are essential for bacterial growth. It is known that the combination of fever and low plasma zinc and iron concentrations helps the host to survive during acute infections (Bullen 1981). Iron is essential for bacterial growth and bacteria compete with host tissue for bound and free iron. Therefore, decreasing the availability of iron, could curtail the multiplication of the organism. A further benefit to the host of a reduction in circulating iron may be to reduce leukocyte-derived toxic oxygen metabolites which are a major contributing factor to the lung lesions found in BPP (Slocombe 1985). Iron plays a major role in the generation of these toxic oxygen radicals and therefore a reduction in iron

availability during the APR may reduce neutrophil dependent tissue injury (Slocombe *et al.* 1990).

Hypoferraemia is thought to be due to redistribution of plasma iron during inflammation with an increase in liver uptake and storage by incorporation into ferritin (Kushner 1982; Koj 1985). This iron is not available for haematopoiesis (Feldman *et al.* 1981a) and may therefore lead to anaemia in chronic infections. A hypoferraemic state during chronic infections has been described in humanS (Cartwright 1966), goats (van Merit *et al.* 1992) and dogs (Feldman *et al.* 1981a).

Injury and infection are often accompanied by haemolysis which may lead to further loss of iron. Haptoglobin (Hp), a major acute phase protein in cattle is also involved in recovery of iron as it binds haemoglobin stochiometrically and irreversibly. This complex (Hp-Hb) retains peroxidase activity and is cleared rapidly from the plasma by the liver. This action of Hp as an effective and specific scavenger is important in bacterial diseases as Hb-iron is known to support bacterial growth (Koj 1985).

Davies *et al.* (1992) reported that iron limitation by the host is influenced by the outer membrane proteins (OMPs) of *P. haemolytica* bacteria and different *P. haemolytica* isolates can have different OMPs (Ali *et al.* 1992). As OMPs are immunogenic, a clear understanding of the relationship between the OMP composition and the host defence could be useful for the production of new vaccines.

In the present study, serum zinc concentrations fell significantly 24 hours post-inoculation. Similar results have been documented in other domestic and laboratory animals (Corrigall *et al.* 1976;. Boosman *et al.* 1989; Kampschmidt and Pulliam 1978). An APR in the horse caused a sharp fall in serum zinc concentration one day after adjuvant administration (Auer *et al.* 1989) and foals experimentally infected with *Rhodococcus equi* became hypozincaemic in comparison with healthy controls (Barton and Embury 1987). A reduction in plasma zinc concentration has been reported in mice following endotoxin injection

and in rabbits after an intra-cranial injection of IL-1 (Fleck and Myers 1985; Turchik and Bornstein 1980). In contrast, serum zinc concentration in dogs increased by 100 per cent after inflammation induced by adjuvant, but this was measured 10 days after treatment (Feldman *et al.* 1981b) and may have followed an immediate drop in serum zinc.

The hypozincaemia observed during the APR occurs when zinc is taken up from the plasma and deposited as metallothionein-bound zinc in the liver (Pekarek *et al.* 1972). Metallothionein is a liver protein, the synthesis of which can be induced by stress, glucocorticoids and administration of metals such as zinc, copper and cadmium (Koj 1985). However, liver uptake of zinc may be dependent on several other factors because in the early stages of the APR the immediate fall in circulating zinc precedes the synthesis by the liver of metallothionein (Koj 1985; Schroeder and Cousin 1990). As these changes occur rapidly, it is also possible that the central nervous system is involved in the increased uptake of zinc by the liver or that cytokines may be involved in the redistribution of zinc in the early stages of the APR (Kampschmidt and Pulliam 1978).

Circulating copper did not change in the infected group until day 5. The increase in copper in the control group on day 1 may have been caused by the contamination of food or water with copper. The increase in copper in the infected group was probably related to the increase in ceruloplasmin which is the major form of copper in the circulation and which has been observed previously in BPP (Conner *et al.* 1989). The mean serum ceruloplasmin in Group I was one of the last acute phase proteins to increase and comparison of Fig. 4.7b with Fig 4.4a indicates that the rise in serum Cu reflects the rise in the acute phase protein ceruloplasmin. In dogs with an adjuvant-induced abscess (Feldman *et al.* 1981b; Fisher 1977), the plasma copper concentration increased on day 10 when the abscesses were opened and drained. Similarly adjuvant stimulated horses showed an increase in copper concentrations from the day 9 to 36 and in these horses

ceruloplasmin levels started rising from day 5 onwards (Auer *et al.* 1989). Furthermore, increased serum copper concentrations have been reported in human rheumatoid arthritis patients (Lunec *et al.* 1981). In the present study a continuing rise in copper (and ceruloplasmin) was not observed because of the short duration of the experimental period.

The rise in serum cortisol and fall in T_4 during BPP has not been described before and indeed, there have been few studies in cattle of the endocrine involvement with infection (Elsasser 1992). However, a rise in cortisol concentration following bacterial infection has been described in other species such as human (Finlay and McKee 1982), mice (Besadovsky *et al.* 1986) and lactating cows (Shuster *et al.* 1993). The mechanism for these hormonal changes during the APR is a subject of much debate. Infection, injury or inflammation can induce the hypophyseal-adrenocortical system to release corticotrophin releasing factor (CRF) to which the pituitary responds with the release of ACTH which, in turn, activates the adrenal cortex to increase glucocorticoid release. In addition cytokines too play a role by stimulating the adrenal cortex directly to release cortisol. Both direct and indirect stimulation of the adrenal cortex will lead to a rise in cortisol concentration (Whicher and Westacott 1992). Besedovsky *et al.* (1986) showed that recombinant human-IL-1β increased ACTH and cortisol concentration in mice. Further studies of corticotropic cells from pituitary incubated with IL-1 confirm that it is a potent inducer for ACTH. *In vivo* increases in ACTH and corticosterone in response to IL-1 were blocked by antibodies to CRF (Sapolsky *et al.* 1987). It has also been reported that IL-1 is able to induce corticosterone release directly from adrenal gland cells in the rat (Salas *et al.* 1990a; 1990b). More recently it has been shown that rh-IL-6 is more potent in this respect and it synergizes with low concentrations of ACTH (Salas *et al.* 1990b; Smith *et al.* 1986).

In this study in Group I the circulating cortisol concentration rose within hours after induction of the acute response and gradually increased till the end of the experiment. The hypophyseal-adrenocortical response was similar for both Groups over the first day of the infection. However, after the second day calves in the Group II began to recover and their cortisol concentrations decreased.

In contrast, to the generally accepted function of cortisol during the APR in its role as a stress hormone, there is still considerable doubts about the cause and function of the fall in thyroxine during the APR. Thyroxine concentrations showed a dramatic fall on day 2 following infection and gradually rose thereafter. In acutely ill human patients with non-thyroidal diseases, low serum concentrations of total T_4, T_3 and thyroid binding globulin (TBG) concentrations are found (Kaplan *et al.* 1982; Wong and Hershman 1992). The main mechanism responsible for the low concentration of T_3 in non-thyroidal illness is a decrease in the peripheral conversion of T_4 to T_3 (Wong and Hershman 1992), whereas the cause of the decrease in T_4 concentration remains speculative. One possibility is that it may be due to low TBG, or the presence of a circulating inhibitor that inhibits T_4 from binding to its carrier proteins (Kaptein *et al.* 1987). Another possible cause of low total T_4 is that decreased biological activity of TSH during acute infections may reduce the secretion of T_4, but as the half-life for T_4 in cattle is 8 days, the sharp drop found in this investigation within 24 hours of infection can not be solely due to reduced production.

Depletion of tissue nutrients during the catabolism of illness can also be the basis of low T_4, T_3 and TBG (Chopra *et al.* 1979). Recent evidence has indicated that acute phase cytokines like TNF_α can inhibit thyroid function directly and may be responsible for the changes in the pituitary-thyroid axis as large amounts of TNF_α receptors are located in the thyroid gland (Wong and Hershman. 1992). However, to date, no mechanism has been accepted as fully explaining the low T_4 state during APR.

Although previous studies on the local response to BPP have demonstrated increased presence of leukocytes in the lung (Dalgleish 1989) this is the first report of systemic leukocytosis in this condition. The cause of leukocytosis was clearly due to a rapid increase in neutrophil counts on day 1 which fell back to normal levels by day 3. This phenomenon is further discussed in Chapter V in relation to the similar response observed following LPS injection.

In this study the pattern of APP to infection showed results in agreement with the previous work reported by Conner *et al.* (1989). Using the ELISA method described in Chapter III bovine SAA was shown to be a major acute phase protein in BPP supporting the previous study (Boosman *et al.* 1989) where SAA was shown to respond to *E coli* LPS. These findings are similar to those reported in man (Sipe 1992), hamster (Niewold and Tooten 1991) and horse (Pepys *et al.* 1989). Bovine SAA was revealed as being equivalent to Hp as an APR, reaching a peak of concentration on the second day after infection. In species, such as man, where Hp is a moderate rather than major APP, SAA demonstrates a more rapid response to stimulation. It would require more frequent blood sampling to establish whether SAA is more sensitive to stimulation than Hp in bovine species.

Fibrinogen is commonly assayed by its coagulating activity in plasma and was determined in these studies only for isolate SC/82/1. It was not surprising that Fb was identified for the first time as an APP in BPP. Although Fb is widely used in veterinary clinical diagnostic work, it only shows a moderate response in cattle (Kent 1992). However Fb in animals with BPP, showed a clear acute response. Unfortunately this protein was not analysed in animals infected with the more virulent isolate (SB/82/1) and it remains unknown whether Fb response may help differentiate between the 2 isolates.

Glutamate dehydrogenase activity was reduced significantly after the *P. haemolytica* infection. Generally plasma GLDH activity indicates that the

liver is damaged and hepatocyte destruction leads to an increase of this enzyme in serum. The reasons for the observed reduction is not known but could relate to the increase in protein synthesis in the liver for the production of the APP. Increased bilirubin levels can indicate biliary obstruction or increased destruction of RBC and the latter would relate to the fall in RBC count found in Group II.

Despite the weight loss that follows an inflammatory insult, the overall host non-specific response to infection and injury is beneficial and is aimed at reducing tissue damage and to avoid secondary bacterial invasion. The similarity of the host response to dissimilar exogenous stimuli have led to the conclusion that it is the local and systemic release of cytokines by monocytes and macrophages that is responsible for the initiation and regulation of inflammation patho-physiological response to BPP infection. However, additional studies are required to characterised the cytokine involvement with BPP and the response of the host to this infection (see Chapter V).

Both of the isolates of *P. haemolytica* used in the investigation (SB/82/1 and SC/82/1) contain similar types of LPS and are both known to be highly virulent (personal communication Dr. R.L. Davies). In this study the APR produced by the two isolates of *P. haemolytica* (SB/82/1 and SC/82/1) was measured independently in order to compare the pathogenicity of the two isolates. The elevation of body temperature is a classical feature of the APR but was clearly demonstrated only by the SB/82/1. Furthermore calves given the SB/82/1 isolate exhibited a marked anorexia, apathy and dramatic drop in the live weight. In contrast SC/82/1 did not show any weight loss although food intake was slightly reduced. Among the APR measured in both experimental groups, serum amyloid A concentrations showed a clear difference between the two isolates. Cortisol concentrations were raised at the early phase of infection with both isolates but after day 2, isolate SC/82/1 infected calves of Group II showed a lower cortisol response. Thyroxine concentration dropped in both groups but, the Group I calves showed a greater reduction than Group II. However, haptoglobin and the

respiratory rate showed a similar response in both groups. It is probable that the differences observed, which show that SB/82/1 caused a more severe APR than SC/82/1, are related to the extent of the pulmonary pathology. The pulmonary score and the lung lesions showed a mark difference in the two isolates with isolate SB/82/1 having a more marked local and systemic response. Possibly SB/82/1 had a higher leukotoxic and\or endotoxic effect than SC/82/1. However, more controlled experiments are required to confirm this hypothesis. In future studies to compare different isolates *in vivo* it would be important to have less variations within the animal groups; i.e. use the animals from the same source, with similar pre-infection live weights and produce one infection at the same time. It is interesting that while most acute phase reactants gave a greater response to SB/82/1 (Group I) there were exceptions. Thus Hp and respiratory rates were raised to the same extent in both infected groups. The reason for these differences could be related to differential responses to the mediators of the inflammatory reaction but a full explanation would be the subject of further investigations.

CHAPTER V

THE IMMEDIATE ACUTE PHASE RESPONSE IN BOVINE PNEUMONIC PASTEURELLOSIS: CYTOKINES, LIPOPOLYSACCHARIDE AND ACUTE PHASE PROTEINS

5.1. Introduction

An understanding of cytokine action and the host responses to bacterial infections has been obtained from *in vitro* studies and from experiments using laboratory animal models in which a wide range of substances including live bacteria, lipopolysaccharide (LPS) and recombinant cytokines had been introduced directly into target tissue cultures or administered intravenously into animals. Although the effects of cytokines have been studied extensively in laboratory animals (Heinrich *et al.* 1990; Kunkel and Remic 1992) and man (Kishimoto 1989) very few studies have been carried out in cattle or in other domestic animals.

The cytokines interleukin-1 (α and β), interleukin-6 and tumour necrosis factor$_\alpha$ are important early mediators of the acute phase response. In cattle, IL-1 activity in parturient paresis has been studied in relation to its ability to induce osteoclast activity (Goff *et al.* 1992). These workers demonstrated that an injection of IL-1β to cows in late pregnancy reduced the incidence of parturient paresis. In another study, Collins *et al.* (1989) using a bovine thymocyte cell culture, measured IL-1 like activity in cattle with intra-mammary devices. Using recombinant bovine IL-1 as standards they showed that IL-1-like activity in mammary macrophages increased with the tissue damage caused by the devices. In a more recent study, Winstanley and Eckersall (1992), using a D-10 mouse T-lymphocyte mononuclear cell line, demonstrated IL-1 like activity in supernatant from bovine mononuclear cells stimulated with LPS and that the IL-1 like activity was dependent on the dose of LPS. In addition, these workers found that the bovine IL-1 like activity could be neutralised by anti-human IL-1α and IL-1β antibodies.

Stressful events such as parturition, lactation, nutritional deficiencies and environmental changes may induce immunosuppression and increase disease susceptibility in cattle (Griffin 1989). Administration of recombinant cytokines such as IL-1 or granulocyte colony stimulating factor (G-CSF) activates of lymphocytes as well inducing other cytokines required

for stimulation of immune cells, emphasising their potential value as immunomodulatory agents (Stabel *et al.* 1991). In a later study, Stabel *et al.* (1993) injected recombinant bovine-IL-1β to 6 Jersey cows (166 ng of rb-IL-1β per kg of body weight) at 8 hour intervals for 96 hours. Leukocytosis was observed within 3 hours of rb-IL-1β treatment and peaked within 24 hours after the first injection and returned to the base line after 72 hours. Within 24 hours of the first rb-IL-1β injection, rb-IL-1β mRNA transcription in stimulated blood mononuclear cell cultures was markedly increased, suggesting that rb-IL-1β upregulates its own production in mononuclear cells. In another study (Shuster *et al.* 1993) 6 cows had 10 μg of *E-coli* LPS infused into 1 mammary gland. Endotoxins caused udder oedema 1.5 to 2.5 hours after the infusion, high milk somatic cell counts and all treated cows showed increased cortisol levels within 3-5 hours. High concentrations of IL-1 (10 - 600U ml^{-1}) and IL-6 (2 to 22 U ml^{-1}) were detected in milk of infused glands from 2.5 to 4 hours after infusion, whereas TNF_{α} was undetectable. Systemic responses and the leukocytic influx into endotoxin-infused glands developed concurrently with the initial cytokine activity. However, at present little is known on the relationship between the acute phase response (APR) and IL-1 activity *in vivo* in cattle.

There have been a number of studies on bovine TNF_{α} (Kenison *et al.* 1991; Pace *et al.* 1993; Espinasse *et al.* 1993). The *in vitro* release of TNF_{α} from bovine peripheral blood monocytes and alveolar macrophages has been demonstrated following stimulation by *E. coli* LPS (Adams and Czuprynski 1990).

The concentration of tumour necrosis factor in the serum of calves after a challenge of *S. typhymurium* and LPS from *E coli* has been investigated by Peel *et al.* (1990). In this study TNF_{α} levels began to rise 30 minutes after administration of LPS, reached peak levels between 1-2 hours and returned to baseline by 4 hours after administration. However, calves infected with *S. typhymurium* failed to show a TNF_{α} response in spite of demonstratable

septicaemia. In another *in vivo* study Kenison *et al.* (1991) demonstrated a similar rise in TNF_{α} following the administration of *E-coli* LPS. Recently, two groups of workers reported conflicting results from experiments designed to study the systemic TNF_{α} response in calves infected with *P. haemolytica.* Pace *et al.* (1993), using a bioassay, demonstrated an increase in circulating TNF_{α} following intra-tracheal inoculation of *P. haemolytica.* The rise in the TNF_{α} concentration was observed within 2 hours of challenge and lasted for 48-72 hours. In contrast to these observations Espinasse *et al.* (1993), using a radioimmunoassay, were unable to detect any increase in serum TNF_{α} concentrations in calves inoculated with *P. haemolytica* by combined intra-nasal/intra-tracheal route. In the latter study, the TNF_{α} concentrations were measured in serum collected at 6 hourly intervals over a period of 3 days after treatment.

Interleukin-6 (IL-6) is another multifunctional cytokine which is known to play an important role in the early APR and is a major stimulant of the acute phase protein (APP) synthesis by hepatocytes. Furthermore, it stimulates the adrenal cortex to release glucocorticosteroids which also have an effect on acute phase protein production (Le and Vilcek 1989; Heinrich *et al.* 1990). However, there have been no reports of IL-6 or IL-6 like activity in bovine serum. Nakajima *et al.* (1993) monitored the APP response in calves following intravenous injection and continuous infusion of recombinant human IL-6 (rh-IL-6). These studies demonstrated an increase in serum haptoglobin and fibrinogen concentrations after rh-IL-6 was infused over a long period but not when rh-IL-6 was administered as a single intravenous injection.

Work described in Chapter 4 demonstrated that a wide-ranging systemic APR occurs during bovine pasteurellosis. However, to understand the initiating factors of the host's response to BPP it is necessary to study the immediate APR in order to determine the extent of cytokine involvement in this stage of the disease. The cytokines which initiate the APR are released immediately after the insult

(Vogel and Hogen. 1990) and as these cytokines have a short half-life (Waage *et al.* 1992) frequent sampling during the early stages of the APR must be employed to detect peaks in the cytokine concentration (Peel *et al.* 1990).

The induction of an APR by Gram-negative bacteria is believed to be caused by stimulation of monocytes and macrophages by the LPS. The LPS of *P.haemolytica* has been characterised recently (Davis *et al* 1992) and shown to vary between field isolates. Hence this second *in vivo* study was undertaken to determine whether LPS from *P.haemolytica* was capable of stimulating cytokine release *in vivo* and initiating the immediate cytokine network and the subsequent systemic APR. In this experiment a single sublethal dose of LPS was administered intravenously and blood samples were collected at short intervals for the first 10 hours after the challenge and then less frequently over the next 5 days.

The aims of the study

a) To determine whether the cytokines TNF_{α}, IL-1 and IL-6 are mediators of the immediate systemic acute phase response in bovine pasteurellosis.

b) To determine whether APP and haematological response occurs during the immediate APR after LPS administration.

c) To determine whether the LPS extracted from *P. haemolytica* type A 1 is able to stimulate TNF_{α} production *in vivo* in calves.

d) To determine whether intravenous challenge of LPS from *P. haemolytica* A1 is capable of stimulating an acute phase response similar to that caused by the whole bacterium when inoculated intra-tracheally.

5.2. Materials and Methods

5.2.1. Animals and Experimental Design

All calves were housed in loose boxes and managed as described previously (section 4.2.1). Nasopharyngeal swabs were taken from all calves on several occasions prior to inoculation and examined for the presence of *Pasteurella* spp. as described in section 4.2.2.

Experiment I was performed to achieve aims a) and b) while Experiment II was designed to achieve aims c) and d). These studies were designed to add to our knowledge of the early stages of the APR and differed from the previous studies (Chapter 4) by the high frequency of blood collection over the first 10 hours of the response and the use of a single inoculum of *P. haemolytica* to infect the animals.

Experiment I

A total of nine calves (6 male and 3 female) were used in this experiment. The animals were divided into two groups consisting of 5 calves in the control group and 4 calves in the infected group. The experimental design and the parameters examined are summarised in Table 5.1.

Each calf in the infected group was challenged intra-tracheally (Section 4.2.2) with a single dose of a 3 hour log phase culture of a field isolate of *P. haemolytica* type A1 (4×10^{12} cfu ml^{-1}; 20 ml $calf^{-1}$). The bacterial isolates used in this experiment are summarised in Table 5.2. All calves in the control group received 20 ml of sterile PBS by the same route.

Experiment II

In this experiment, 5 calves (4 males and 1 female) were infused intravenously with a single dose (10 ml) of LPS at 0.7 $\mu g\ kg^{-1}$ extracted from 5 different field isolates of *P. haemolytica* type A1 (Table 5.4). In a trial experiment with 1 calf (No. A1) LPS at 1 $\mu g\ kg^{-1}$ was used but found to be too high for subsequent use.

Table 5.1 Experiment I-The experimental design

	Control Group	Infected Group
No. of calves	5	4
Age	3 months	3 months
Body Wt.(Kg)	131 ± 6	127 ± 8
Infection procedure	Sterile PBS(I/T)	*P. h*-bacteria (I/T)
Challenge (20 ml) per calf	-	4×10^{12}(cfu ml^{-1})
Sampling interval, post-challenge hours	-1, 0,1, 2, 4, 6, 8, 10	-1, 0,1, 2, 4, 6, 8, 10
Clinical Examination	Rec.Tem. Resp.Rate	Rec.Tem. Resp.Rate
Cytokines measured	IL-1; IL-6; TNF_α	IL-1; IL-6; TNF_α
Acute phase proteins	SAA; Hp	SAA; Hp

I/T - intra-tracheal, SAA serum amyloid A, Hp haptoglobin, IL-1 interleukin-1, IL-6 interleukin-6, TNF_α tumour necrosis factor α. Rec.Tem.- rectal temperature, Resp. Rate- respiratory rate, Organism- live *P. haemolytica*

Table 5.2. Details of bacterial isolates used in the Experiment I.

Calf no	Isolate
33	S/C 82/1 Bovine
2	S/B 82/1 Bovine
197	W/D 83/4 Bovine
16T	FA 1 Bovine

All isolates belonged to serotype A1.

Table 5.3 Experiment II - The experimental design

	Control Group	LPS Treated Group
No. of calves	5	5
Age	1 to 2 months	1 to 2 months
Body Wt. (Kg)	110 ± 6	113 ± 10
Challenging procedure	sterile PBS I/V	LPS I/V
Challenge dose	10 ml per calf	0.7 μg kg^{-1}
Sampling interval	0,1, 2, 4, 6, 8, 10 hr and 1-5 days	0,1, 2, 4, 6, 8, 10 hr and 1-5 days
Clinical examination	+	+
APP	SAA; Hp; Fb	SAA; Hp; Fb
Cytokine Assay	TNF_{α}	TNF_{α}

I/V - intravenous; SAA-serum amyloid A; Hp-haptoglobin, Fb-fibrinogen;

Table 5.4. Experiment II-Details of endotoxin extracts and the dosage per animal

Calf no/sex	Body Wt. (kg)	Strain	Total LPS dose (μg)	μg LPS kg^{-1} B. Wt.
OX1/M	120	PH 30	84	0.7
OX2/F	120	PH 8	84	0.7
21T/M	92	PH44	64	0.7
8/M	78	PH 2	54.5	0.7
12T/M	155	B664	108.5	0.7

Control calves (n=5) were infused with 10 ml of sterile PBS. The details of animals used and parameters examined are given in Table 5.3.

The animals used in this experiment were part of a pilot study for an associated project on the pathology induced by LPS from different field isolates of *P. haemolytica* in calves. The LPS samples were prepared by Drs. R.L. Davies and R. Parton, Department of Microbiology, University of Glasgow. The experiment was designed to study the APR of calves to different field isolates of LPS from *P. haemolytica*.

5.2.2. Blood collection

In Experiment I, jugular blood samples (10 ml) were collected from all calves one hour prior to inoculation, and at 0, 1, 2, 4, 6, 8 and 10 hours after inoculation. In Experiment II, jugular blood (10 ml) was collected as in Experiment I but also at 24, 36, 48, 72, 96 and 120 hours after inoculation. Blood for measurement of leukocyte counts was collected in 10 ml vacutainer tubes containing 0.38 mM EDTA. Blood for the determination of cytokines (TNF_{α}, IL-1 and IL-6) was collected in chilled, endotoxin free (Endo Tube ET) vacutainer tubes with no coagulant from Chromogenix AB (Taljegärdsgatan 3, S-431 53 Mölndal, Sweden.). Plasma samples for acute phase protein determinations were collected as described in section 4.2.2. All blood samples (except those for haematology) were centrifuged at 1600g for 20 minutes at 4°C, serum was harvested and stored at -20°C until assayed.

5.2.3. Clinical examination

Calves were observed hourly post-inoculation with *P. haemolytica* and following treatment with LPS. The details recorded at each clinical examination included observations on demeanour, rectal temperature, respiratory rate and character, the presence or absence of cough, nasal and ocular discharges and any other relevant clinical findings.

5.2.4. Acute phase proteins

The concentration of SAA in serum was assayed by ELISA (Horadagoda *et al.* 1993; Chapter 3 (Section 3.2.3.) and the haptoglobin concentration was assayed according to the method of (Conner *et al.* 1988b) described in Section 4.2.10.2. Fibrinogen was analysed as described in Section 4.2.10.3.

5.2.5. Analysis of cytokines

5.2.5.1. Radioimmunoassay for bovine Tumour Necrosis Factor$_a$

Bovine TNF_α was measured using a competitive, sequential-saturation, non-equilibrium radioimmunoassay. In order to increase the sensitivity of the assay non-equilibrium conditions were employed by delaying the addition of the ^{125}I labelled antigen. The RIA was a modification of the method described by Kenison *et al.* (1990) and was performed under the supervision of Dr. J C. Hodgson at the Moredun Research Institute, Edinburgh.

Reagents

The TNF_α used in the assay was recombinant bovine rb-TNF_α which was produced, cloned and expressed in *E. coli* and purified by HPLC. It was kindly provided by Dr. Gary Entrican, Division of Immunobiology, Moredun Research Institute through the courtesy of CIBA-GEIGY Limited, St. Aubin-FR, Switzerland. The rabbit anti-serum to bovine TNF_α was raised against rb-TNF_α and was kindly provided by Dr. P. M. Preston, (Division of Biological Science, University of Edinburgh, Edinburgh). Donkey anti-serum to rabbit IgG and normal rabbit serum were from the Scottish Antibody Production Unit (Lanarkshire, Scotland). The Sephadex G-25 gel filtration column (PD-10) was obtained from Pharmacia (Uppsala, Sweden). Radiolabelled sodium [^{125}I] iodide was purchased from Amersham International (Amersham, Bucks, UK). Iodogen (tetrachlorodiphenylglycouracil), bovine serum albumin and Tween 20 and other laboratory chemicals were from Sigma Chemical Company (Poole, Dorset, UK). Polyethylene glycol 6000 was from BDH Chemicals (Poole, Dorset, UK) and the

polystyrene Röhren tubes used in the assay were obtained from Ciba Corning Diagnostic Limited (Halstead, Essex, UK).

Assay buffer

All antibody dilutions were prepared in assay buffer which consisted of 0.15M phosphate buffered saline, pH 7.4, with 1% (w/v) bovine serum albumin and 0.1% (v/v) Tween 20.

First antibody

The first antibody was prepared by diluting the rabbit antiserum to rb-TNF_{α} 1:250 in assay buffer.

Second antibody

The second antibody was prepared by mixing 2 parts of a 1:40 dilution of the donkey anti-serum to rabbit IgG with 7 parts of a 6% (w/v) solution of polyethylene glycol 6000 in assay buffer.

Iodination Procedure

An iodinated tracer of rb-TNF_{α} was prepared by the iodogen method described by Elsasser *et al.* (1986). rb-TNF_{α} (10 mg) was allowed to react with 0.5 mCi of [^{125} I] in 95 ml of borate-buffered saline (pH 8.4) in a tube previously coated with 7.5 mg of iodogen. Unbound iodine was removed by passing the mixture through a Sephadex G25 gel filtration column pre-equilibrated with borate buffered saline containing 0.2% (w/v) bovine serum albumin and 5% (v/v) Tween 20. The average specific activity of the tracer produced by this procedure was 60 mCi mg^{-1}; a dilution in assay buffer which yielded approximately 10, 000 CPM per 100 µl was used in the assay.

Standards

Standards were included in each assay and were prepared by diluting a stock solution of rb-TNF_{α} to give concentrations of 500, 100, 25, 5, 1 and 0.25 ng ml^{-1} in foetal bovine serum (FBS).

Assay validation

Quality Control Samples

A high (250 ng ml^{-1}) and a low (5 ng ml^{-1}) quality control sample of bovine TNF_{α} were prepared by spiking foetal bovine serum with rb-TNF_{α} and used to determine intra-assay coefficients of variance. The limit of detection was calculated as the concentration of TNF_{α} at two standard deviations from the zero serum which was foetal bovine serum. The radioimmunoassay for TNF_{α}, was performed according to the method described by Kenison *et al.* (1990; 1991) and was validated for the analysis of bovine plasma. In the present study, the limit of detection of the assay was 250 pg ml^{-1} and the intra-assay coefficients of variance were 6% at 250 ng ml^{-1} and 17 % at 5 ng ml^{-1}.

Assay procedure

The assay was performed in 75 x 11 mM Röhren tubes. Fifty microlitres of the standards, controls or test sera, in duplicate, were mixed with 50 µl of first antibody (1:250 dilution) and 50 µl of assay buffer in the assay tubes. After vortex-mixing the tubes were incubated at 4°C for 24 hours to allow antigen in the samples and the first antibody to react. At the end of this period, 50 µl of [^{125}I]-labelled-rb-TNF_{α} was added to all the tubes, vortexed and incubated for a further 18 hours at 4°C. Tubes with the labelled TNF_{α} alone were incubated to provide the total count of tracer. The second antibody (200 µl) was then added to all the tubes, apart from the tubes for the total count of tracer, and incubated for 2 hours. To enhance pellet formation, 50 µl of 1% (v/v) normal rabbit serum was added to the tubes and the incubation continued for a further 30 minutes. All tubes were then centrifuged (12,000 g 6 min; Centra 3S, Damon/IEC), the supernatant aspirated and the radioactivity of the pellet measured in a gamma counter (Packard Auto-Gamma-5650, Pangbourne, Berks, UK). The results were analysed using the S. A .S. immunoassay program 632014 written and supplied by Dr. P. Edwards, Dept. of Molecular Endocrinology, UCMSM, London, UK.

5.2.5.2. Assay for Interleukin 1 (IL-1)

Biological basis of IL-1 assay

IL-1 was estimated by a biological assay developed by Collins *et al.* (1989) using bovine thymocytes and with recombinant bovine IL-1 (rb-IL-1) (Collins, R.A. personal communication) as standard. In this assay, the bovine thymocyte cells depend on IL-I for proliferation which was low with control media but increased up to 10 fold in the presence of IL-I and by 4 fold when the mitogen concanavalin A was added at 5 $\mu g\ ml^{-1}$. The assay was performed at the Institute of Animal Health, Compton, (Newbury, Berks) under the supervision of Dr. R. A. Collins. The limit of detection of the assay was 0.1 $pg\ ml^{-1}$.

Reagents for the IL-1 assay

All reagents used were of the molecular biology grade. Recombinant bovine IL-1 was kindly provided by Dr. R. A. Collins. Culture medium RPMI 1640 was obtained from Microbiological Associates, (Walkersville, MD). Concanavalin A (con. A) a mitogen and Histopaque for cell separation were obtained from Sigma Chemical Company (Poole, Dorset, UK). Foetal bovine serum and flat-bottomed microtitre plates were obtained from Gibco (Uxbridge, Middlesex, UK). ^{3}H-Thymidine (specific activity 5Ci mM^{-1}) was purchased from Amersham International (Amersham, Bucks, UK).

Preparation of thymocytes

Thymus tissue was collected from a clinically healthy calf at slaughter. The thymus was initially cleaned of fatty or fibrous tissues before macerating in a "stomacher" (Institute for Animal Health, Compton, Berks) with 20 ml of 0.01M phosphate buffered saline (PBS; pH 6.8). The resultant cell suspension was diluted further with PBS (1:4; v/v), transferred to 50 ml plastic universal tubes (Sterilin Ltd, Feltham, Middlesex, UK) centrifuged at 900 x g for 20 minutes at 6°C and washed three times with PBS. Thymocyte cell suspension (10 ml) at 5 x 10^{9} cell ml^{-1} was layered over 8 ml of Histopaque (density 1.083) and centrifuged at 900g for 20 minutes at 4°C. After centrifugation, cells at the

interface were collected with a sterile pipette, washed three times in 0.01M PBS (15ml per wash) and resuspended at a concentration of 4 x 10^7 cell ml^{-1} in tissue culture medium (TCM-1) which consisted of RPMI 1640 medium with 10% (v/v) heat-inactivated foetal bovine serum, Con A 0.2 mg ml^{-1}, 50 mM 2-mercaptoethanol, 2 mM glutamine, 27 mM sodium bicarbonate and 1mM sodium pyruvate, 200 units ml^{-1} penicillin and 100 mg ml^{-1} streptomycin. The TCM-1 was filtered (0.22 μm; Millipore filter) before use. Cell viability was determined by exclusion of 0.5% (w/v) trypan blue and was judged to be greater than 84% in the preparations used.

Calf thymocyte costimulation assay

Serial two fold dilutions of plasma samples were made in triplicate in microtitre plates using TCM-1 to achieve the following dilutions 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 in 100 μl volumes. A standard of rb-IL-1 was also serially diluted in triplicate to give a range of IL-1 from 0.1 to 100 pg per well. In each plate there were 4 sets of culture controls in triplicate as shown below (Table 5.5).

Table 5.5. Details of the culture controls

Control No	TCM-1	Thymocytes	Con.A	LPS
1	+	+	-	-
2	+	+	+	-
3	+	+	-	+
4	+	+	+	+

LPS= 0.025 μg ml^{-1} lipopolysaccharide (LPS; 026 B6); Con.A= 5 μg ml^{-1}

Calf thymocytes, isolated as described above, were cultured at 4 x 10^6 cells $well^{-1}$ in flat-bottomed, 96-well microtitre plates containing serial two-fold dilutions in TCM-1. Similarly test samples (plasma samples collected as described in 5.2.2.) and standards of rb-IL-1 (0.1-100 Units ml^{-1} i.e. 0.1 to 1000 pg ml^{-1})

were diluted with TCM-1. Three quality control samples were used in the assay at high, (C-I) medium (C-II) and low (C-III) concentrations of rb-IL-1 (3 foetal calf serum samples were spiked with known concentrations of rb-IL-1 at 64.0 pg ml^{-1}; 4.0 pg ml^{-1}; 0.2 pg ml^{-1}). Three serum samples from *P. haemolytica* infected calves were also spiked with 50 pg ml^{-1} of rb-IL-1 and used in the assay as serum controls. The thymocytes were added in a volume of 100 µl per well to the diluted test samples or standards in the wells of the microtitre plate. Cultures were incubated for 72 hr at 37°C in a humidified atmosphere containing 5% CO_2. For the final 6 hours of culture 0.5 mCi [^{3}H]-thymidine (specific activity 5 Ci $mmol^{-1}$) was added. The cultures were harvested on to glass fibre filters using a semi-automatic harvester (Skatron), and the incorporated radioactivity was measured using a liquid scintillation counter (LKB Wallac, Turuk, Finland). Results were recorded as counts per minute (CPM) before conversion to pg ml^{-1} by comparison to the standard curve.

5.2.5.3. Bioassay for bovine Interleukin - 6 (IL-6) using B9 cell-line

Most bioassays for IL-6 depend upon the proliferative effect of this cytokine. The present work used the assay developed by Aarden *et al.* (1987) which makes use of this phenomenon to measure the effect of human IL-6 on a susceptible murine hybridoma cell line (B9). The assay was performed under the supervision of Dr. C. Lawrence, Department of Veterinary Pathology, Glasgow University Veterinary School.

Reagents

The B9 murine hybridoma cell-line was a kind gift of Dr. L.A. Aarden (Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service, Amsterdam). Flat-bottomed microtitre plates were purchased from Costar (Cambridge, UK). Con A and RPMI 1640 (R 7755) medium from Sigma Chemical Company (Poole, Dorset, UK), FBS was from Gibco (Uxbridge, Middlesex, UK), [^{3}H]-Thymidine (specific activity 5Ci.mM^{-1}) from Amersham

International (Amersham, Bucks, UK). Methylthymidine and recombinant human IL-6 (rh-IL-6) from Genzyme Corporation (Cambridge, UK).

Maintenance of the B9 cell-line

The B9 cell line was in RPMI 1640 medium supplemented with 10% (v/v) FBS plus 1 U ml^{-1} of IL-6 (in tissue culture medium-2, TCM-2). Cultures were maintained at 37°C in a humidified carbon dioxide (5% CO_2) incubator. Cultures were split 1:5 to 1:10 every 2-3 days and using fresh TCM (with IL-6) until the cell density reached approximately $5x10^5$ cells ml^{-1}.

Assay procedure

Cells were washed twice in RPMI 1640 medium by centrifugation at 250g for 10 min and resuspended in TCM-2 to yield a concentration of $5x10^4$ cells ml^{-1}. The viability of the cells were determined by exclusion of 1% (w/v) trypan blue.

The assay was performed in 96 well flat-bottom polystyrene microtitre plates (Costar). Initially, 100 µl of TCM-2 was added to all 96 wells of the plate. This was followed by the addition of standards (100 µl of stock human IL-6 in TCM-2 at 16 units ml^{-1}) and test samples (100 µl plasma per well) in triplicate to the wells in the top row of the plate. Serial two-fold dilution of these were made to obtain a final IL-6 standard range from 8 to 0.125 U ml^{-1}. The final dilution of test sample was from 1:2 to 1:128. The assay negative control consisted of 100 µl of TCM without IL-6. In addition Con A at 0.2 µg ml^{-1} in TCM-2 was added to three wells as a positive control. Three additional controls consisted of 2 culture supernatants from feline peripheral blood lymphocytes ($5x10^6$ ml^{-1}) stimulated for 24 hours with either 10 µg ml^{-1} LPS (control 1) or 7.5 µg ml^{-1} Con A (control 2) or one without any stimulant (control 3).

Finally, 100 µl of the B9 cell suspension was added to each well and the plates were incubated for 72 hours at 37°C in a humidified CO_2 incubator. Cells were labelled by incubating for the final 4-6 hours with 25 µl [^{3}H]-thymidine, at an activity of 0.5 mCi per well, prepared by adding 50 µl of a stock solution of 1 mCi ml^{-1} to 2.5 ml of TCM-2. Following incubation, cells were harvested and

the radioactivity counted as described above. The results were expressed as the arithmetic mean ± SEM of counts per minute (CPM) incorporated per culture for each triplicate group. The IL-6 concentrations in the unknown samples were derived by comparison with IL-6 standards using probit analysis at the 50% response of the rh-IL-6 standard.

5.2.7. Haematology

A complete haematological study was carried out as described in Chapter 4 for Experiment II.

5.2.8. Statistical Analysis

Experiment I and II

Results of the *Pasteurella haemolytica* infection and LPS treatment are presented as individual values for TNF_{α} or group means plus or minus the standard error of the mean (SEM). Comparisons between experimental and control groups were made using one way analysis of variance. Results from the analysis of variance were then used in the Newman-Keuls multiple range test to identify treatment differences.

These statistical analysis were conducted using the MINITAB statistical package (MINITAB Inc., State College, PA 16801-3008 USA) and ANIMAL DESIGNS 1, V1.21 5/6 (Data International Services, Glasgow) programmes on an IBM computer (Personal system/2, Model 30). Responses were considered statistically significant when $P < 0.05$.

5.3 Results

5.3.1. Experiment I

5.3.1.1. *Clinical examination*

The most marked clinical change observed following infection was the alterations in the respiratory rate. Figure 5.1a shows the variation with time in the mean respiratory rates of infected and control animal. Prior to infection the respiratory rates of animals in both groups were within the normal range (15-30 per minute).

Following inoculation the calves in the control group showed a transient increase in the respiratory rate soon after the intra-tracheal inoculation of PBS which returned to the normal rate within 6-8 hours (Fig.5.1a). In contrast, the calves challenged with *P. haemolytica* showed a significant ($P< 0.001$) increase of the respiration rate from a baseline rate of 26 ± 3.4 min^{-1} to 66 ± 6.3 within 4 hours post-inoculation. At 10 hours the respiration rate was 80 min^{-1}. Calves in the control group remained bright and alert throughout the experiment but infected calves became dull, lethargic, anorexic and tachypnoiec within 4 hours after inoculation.

Figure 5.1b shows the mean rectal temperatures of the infected and the control animals. Prior to infection, the rectal temperature of calves in both groups were within the normal range (38.2 - 38.6°C). Following inoculation the rectal temperature of the infected calves increased significantly ($p<0.01$) from a baseline value of 38.4 ± 0.4 to 40.2 ± 0.6°C within 6 hours and remained elevated through out the experimental period. In contrast, there were no appreciable changes in the rectal temperatures of control animals.

5.3.1.2. The acute phase protein response

Prior to inoculation the serum SAA concentrations of calves in both the infected group and the control group were at or below the limit of detection of the assay (≤ 3 μg ml^{-1}). As illustrated in Fig.5.2a. the mean SAA concentration in infected animals increased markedly to 18 ± 3.2 mg ml^{-1} at 10 hours post-inoculation. In comparison, the control group did not show any apparent changes in the serum SAA concentration during this period. The mean serum haptoglobin concentration did not show any significant change and remained within the normal range throughout the experiment in both groups.

5.3.1.3. Cytokines

TNF_α

The time-course of induced TNF_α production following infection with *P. haemolytica* is depicted in Figure 5.2b. In all four infected calves, the serum

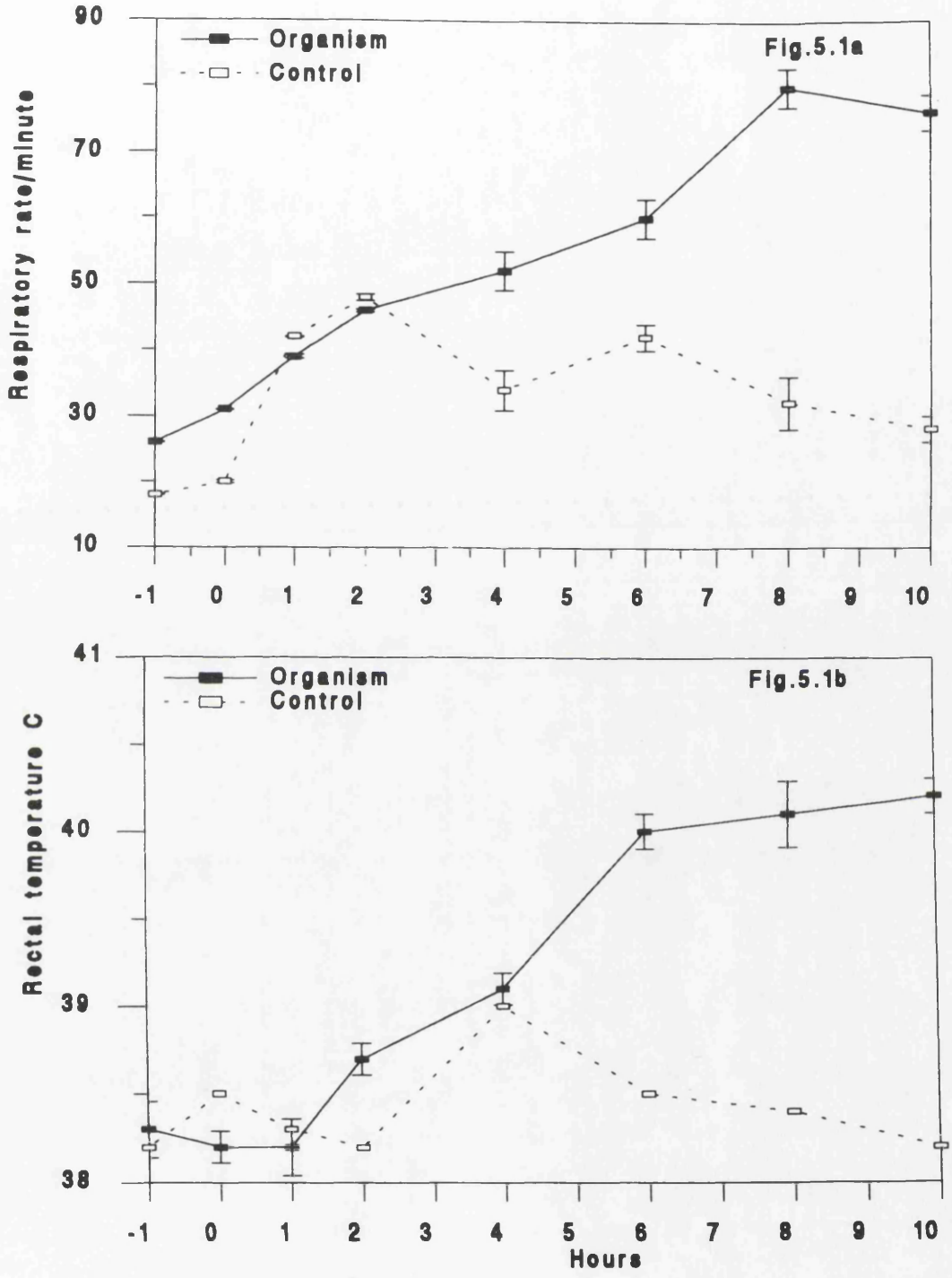


Figure 5.1 Mean (±SEM) respiratory rates (Fig. 5.1a) and rectal temperatures (Fig. 5.1b) over 10 hours of calves infected intra-tracheally with the *P. haemolytica* A1 (n=4). The control calves received phosphate buffered saline intra-tracheally (n=5).

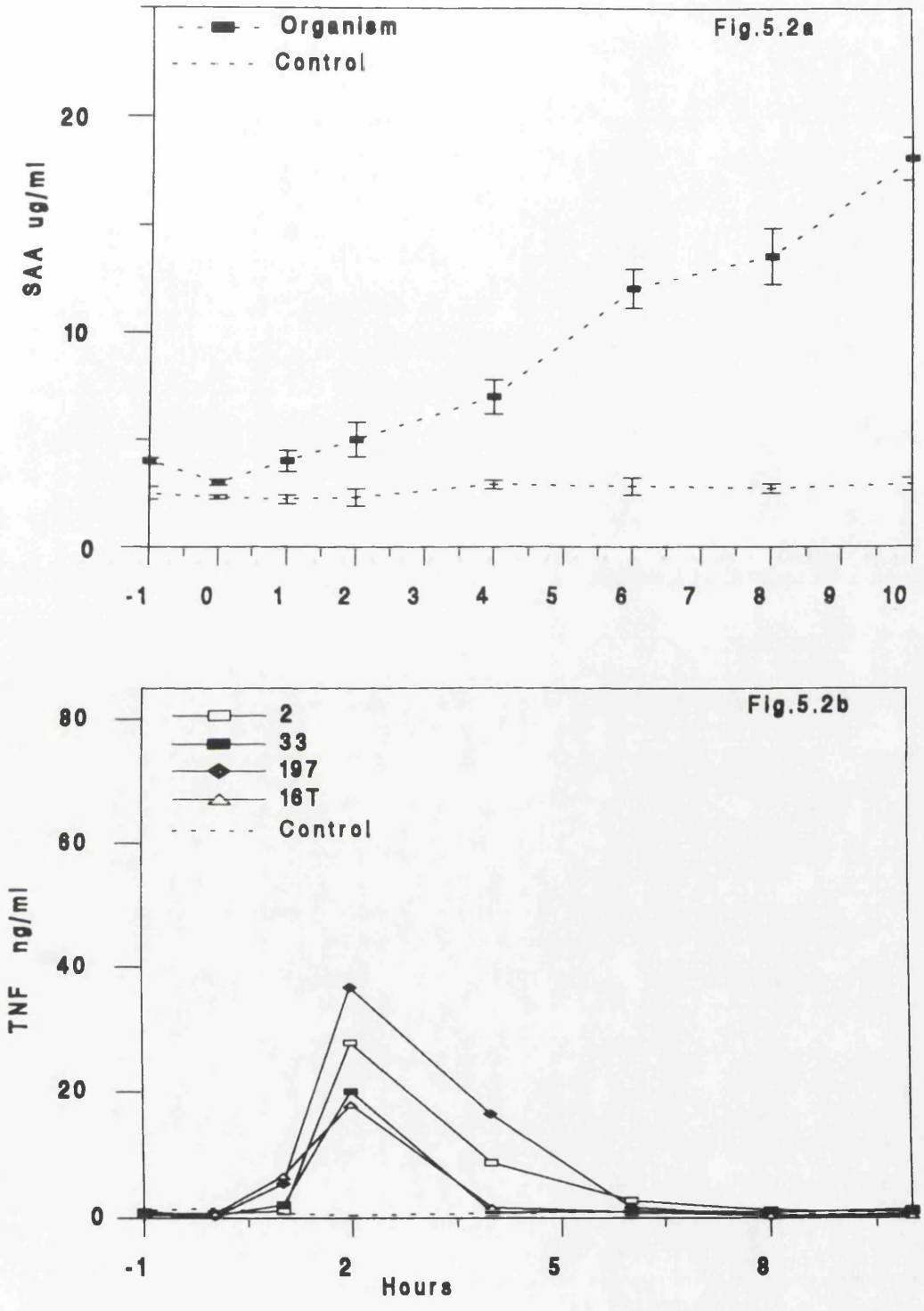


Figure 5.2 Mean (± SEM) serum amyloid A concentrations (Fig. 5.2a) and the individual tumour necrosis factor$_\alpha$ concentrations (Fig. 5.2b) over 10 hours of calves infected with *P. haemolytica* A1, intra-tracheally (n=4). The control calves received phosphate buffered saline intra-tracheally (n=5). In the control group for tumour necrosis factor$_\alpha$ only the mean values are shown.

TNF_{α} concentration rose to a peak at 2 hours post-inoculation, ranging from 18-37 ng ml^{-1}, (mean ± SEM = 25.8 ± 8.7 ng ml^{-1}) and then declined rapidly to reach baseline values at 4-8 hours post-inoculation. TNF_{α} was not detected in serum samples from the PBS-treated control group or in animals prior to infection.

IL-1

Validation of the IL-1 assay used in this study was described by Collins *et al.* (1989). The assay has been used to measure the IL-1 concentration in synovial fluid, peripheral blood lymphocytes (PBL's), and in serum or plasma.

Figure 5.3a shows the IL-1 standard curve for the incorporation of [3H] thymidine. Figure 5.3b. shows the IL-1 content of the assay controls; blank - 1840 CPM, Con. A positive - 5575 CPM and LPS positive - 292398 CPM. In addition, three rb-IL-1 spiked samples which were designated as control-1 (123460 -CPM), control-2 (35678 - CPM) and control-3 (10111 - CPM) were also used as quality controls in the assay. The values obtained for the IL-1 assay standards and the controls agreed with values already established in the laboratory.

However, in the serum obtained from infected animals and in all dilutions, the incorporation of [^{3}H] was at or around the counts obtained with the assay blank. So that the IL-1 levels were undetectable. In order to rule out the possibility that inhibitors in serum were inactivating IL-1, three serum samples (IS-1; IS-2; IS-3) from infected animals were spiked with rb-IL-1 (50 pg ml^{-1}) and measured in the assay. These samples gave counts which corresponded to an IL-1 concentration of 50.4 pg ml^{-1}; 49.6 pg ml^{-1}; 51.0 pg ml^{-1} respectively (Fig. 5.3b).

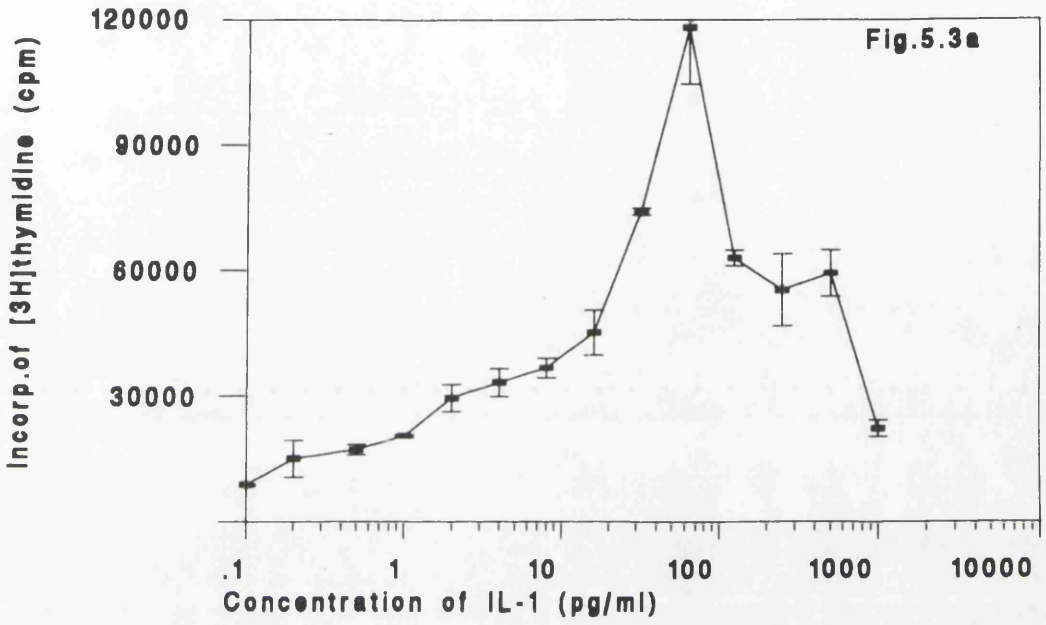


Figure 5.3a Standard curve for recombinant bovine IL-1 from calf thymocyte proliferation assay. Reciprocal dilution of rb-IL-1 from 1000 pg ml^{-1} to 0.1 pg ml^{-1} in culture medium containing 5% foetal calf serum. IL-1 concentration is proportionate to the [^{3}H]-thymidine counts per minutes. Error bars represents the ± SEM of six standards at each point.

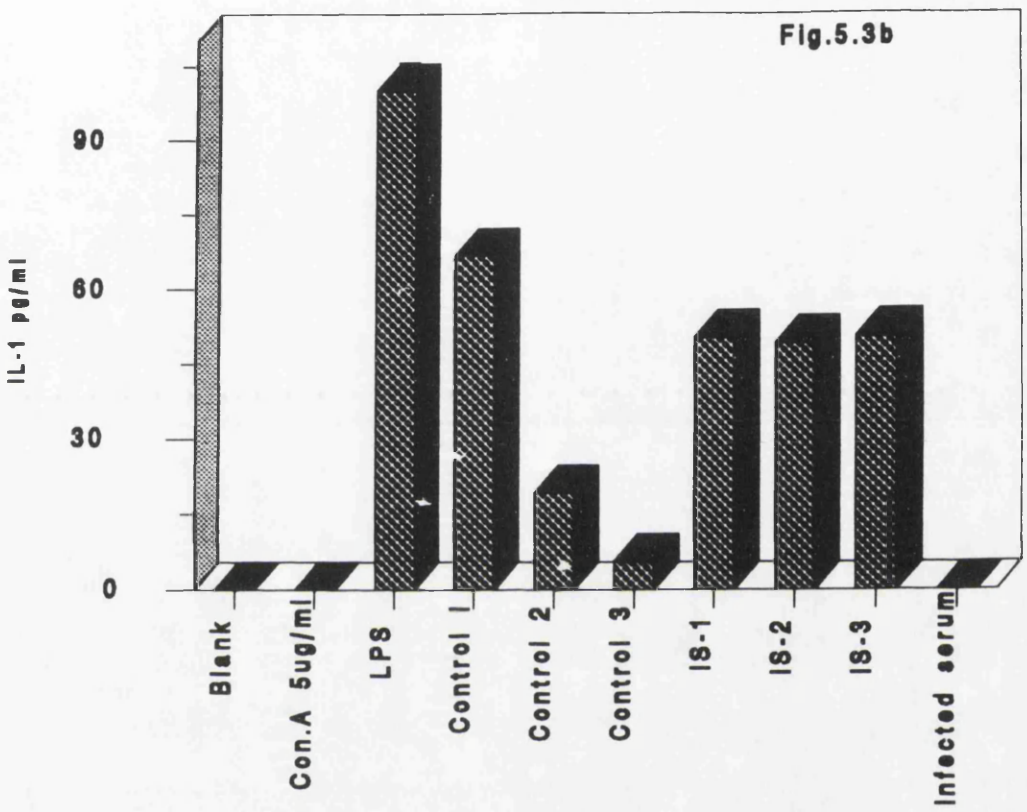


Figure 5.3b Interleukin 1 bioassay controls and sample controls. Histogram represents three assay controls; blank (only the culture medium); Con.A control (culture medium with 5 μg ml^{-1} Con A) and LPS control (culture medium with 0.025 LPS). The culture medium controls with added IL-1 include Control 1 (65 IL-1 pg ml^{-1}), Control 2 (8 IL-1 pg ml^{-1}) and Control 3 (0.2 IL-1 pg ml^{-1}). Three *P. haemolytica* infected calf sera spiked with 50 pg ml^{-1} of rb-IL are represented as IS-1, IS-2 and IS-3. The IL 1 activity in the infected serum sample without spiking was below the assay sensitivity limit at 0.1 IL-1 pg ml^{-1}.

IL-6

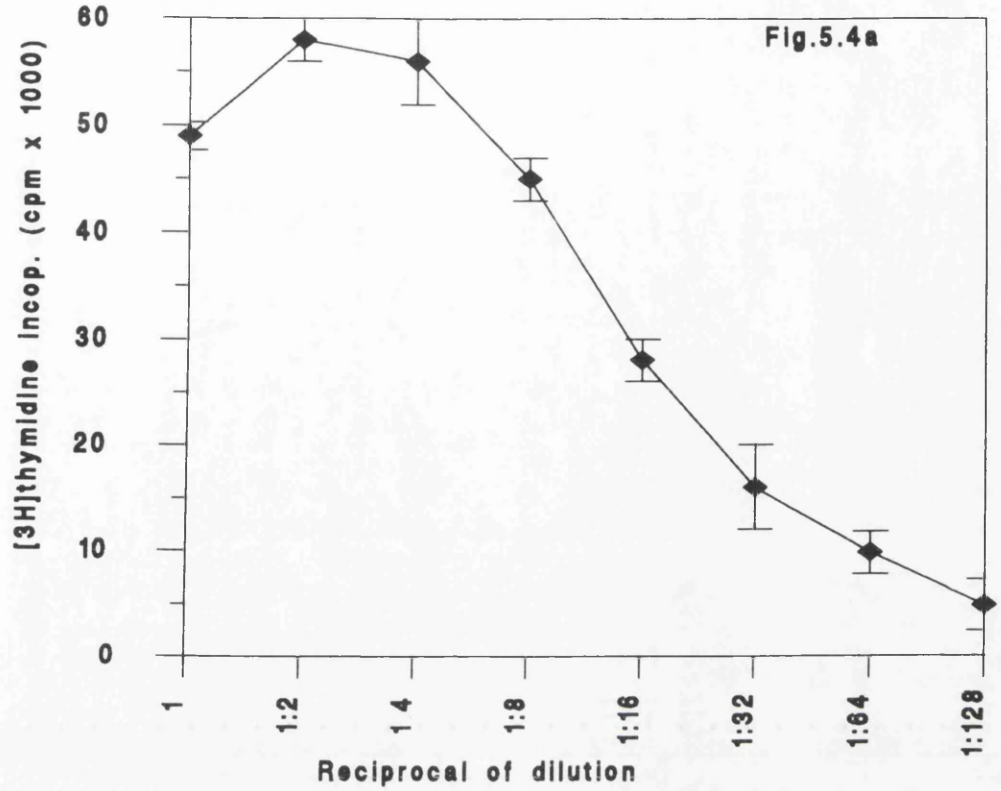
Bovine IL-6 was not detected by the bioassay. The assay standards (Figure 5.4a) and the three controls (Fig.5.4b) with supernatants from cat PBL's stimulated by LPS, Con. A or without any stimulant gave 90; 38 and 17 Units per millilitre IL-6, respectively. However, the wells containing bovine serum gave very low counts which were below the counts in the negative control and could not be interpreted using the standard curve.

5.3.2. Experiment II (10 hour duration)

The acute phase response in LPS-treated calves was monitored for the immediate response over the first 10 hours and the delayed response up to day 5 post infection. The results obtained were compared with Experiment I in this chapter in relation to the immediate response and the experiments described in Chapter IV in relation to the response over following 5 days.

The measurements of the respiratory rates and the rectal temperatures of the LPS-treated and control calves which received intravenous PBS over the initial 10 hours are shown graphically in Figures 5.5a and 5.5b, respectively. None of the calves in the control group were tachypnoeic. On the other hand, LPS treated calves became depressed, anorexic, recumbent and showed a rapid increase in the group mean respiratory rate. In these animals the respiratory rates increased from a baseline value of 18 ± 1.2 per min to 70 ± 2.6 per min, after 1 hour. When compared to the control group there was a significant ($P<0.01$) rise in the respiratory rate but this lasted only about 4 hours and gradually dropped to 38 ± 1.2 per minutes. In contrast to temperature changes observed in Experiment I in which animals were infected with the organism, the calves treated with LPS showed only a slight febrile response which was not significantly different from the controls.

Administration of PBS (control group) did not cause any significant clinical change during the entire experimental period.



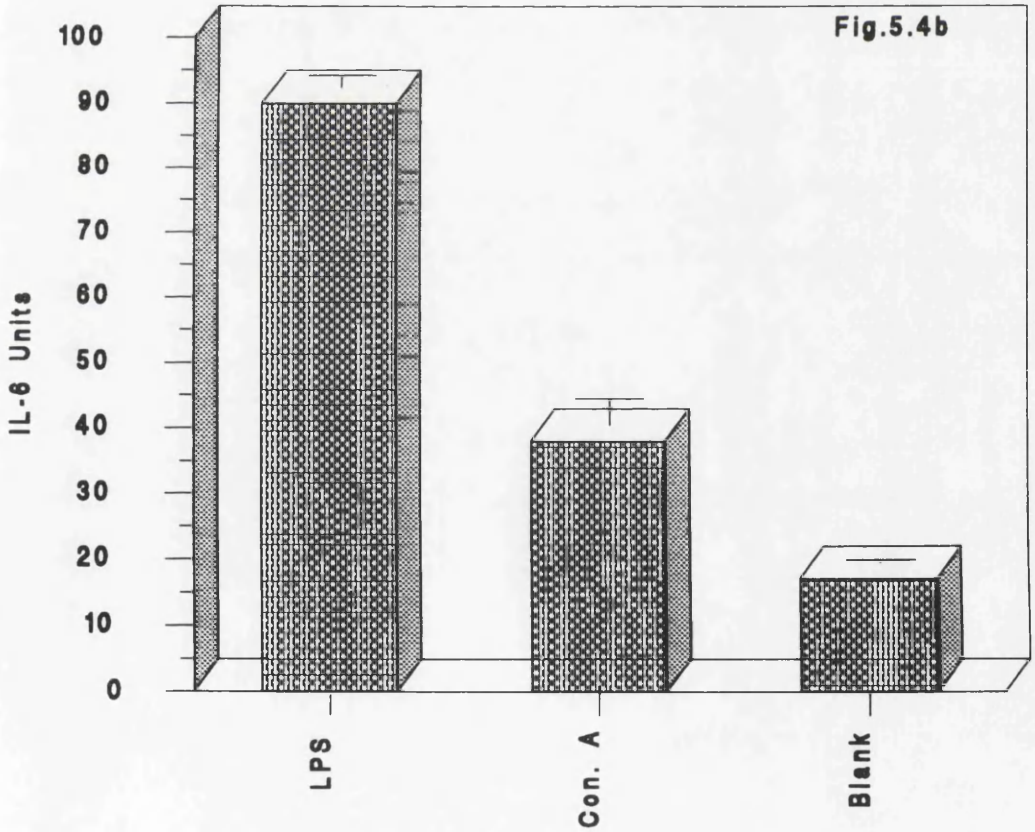


Figure 5.4 Interleukin 6 standard curve (Fig. 5.4a). The histogram (Fig. 5.4b) indicates the IL 6 activity LPS, Con A and a blank used as controls.

5.3.2.1 Acute phase proteins

Following LPS infusion there was a significant ($P<0.05$) rise in the SAA concentration within 6 hours post treatment and at the end of 10 hours the SAA had risen from a pre-treatment value of 3.5 ± 0.3 mg ml^{-1} to 20.2 ± 0.9 mg ml^{-1} (Fig. 5.6a). The serum Hp concentration was 0.2 ± 0.23 mg ml^{-1} before the treatment with LPS and showed a marginal rise to 0.45 ± 0.11 mg ml^{-1} at the end of 10 hours.

5.3.2.2 Cytokines

Due to limitation on the resources for the TNF_{α} assay sera from only 4 infected and 5 control calves were analysed for TNF_{α} activity. The individual serum TNF_{α} concentration in the 4 calves treated with LPS are shown in Figure 5.6b. Of the 4 calves 3 were treated with 0.7 μg kg^{-1} of LPS while the fourth calf (A-1) received a higher dose of LPS (1 μg kg^{-1}). TNF_{α} concentration started to rise significantly ($p<0.001$) 1 hour after the LPS treatment and peaked at 2 hours ranging from 11.33 - 80.24 ng ml^{-1}, (mean ± SEM concentration = 33.8 ± 15.0 ng ml^{-1}). By six hours post-infection, TNF_{α} levels fell rapidly to reach the pre-infection concentration, except for calf A-1 which maintained detectable TNF_{α} in serum till 10 hours post-treatment. Sera from control calves did not contain detectable amounts of TNF_{α} at any time after the injection of PBS.

5.3.3. Experiment II delayed response

5.3.3.1. Acute phase proteins

The mean SAA and Hp concentrations in serum taken over the following 5 days are illustrated in Figures 5.7a and 5.7b, respectively. The LPS-treated group and the control group had barely detectable levels of SAA and Hp prior to treatment. Over the 5 days after inoculation the mean SAA and Hp concentrations increased in the LPS treated group with the concentration of both proteins peaking around 24 hours and then gradually declining thereafter. There was a two-fold rise in the fibrinogen concentration on the day of treatment. This fell to

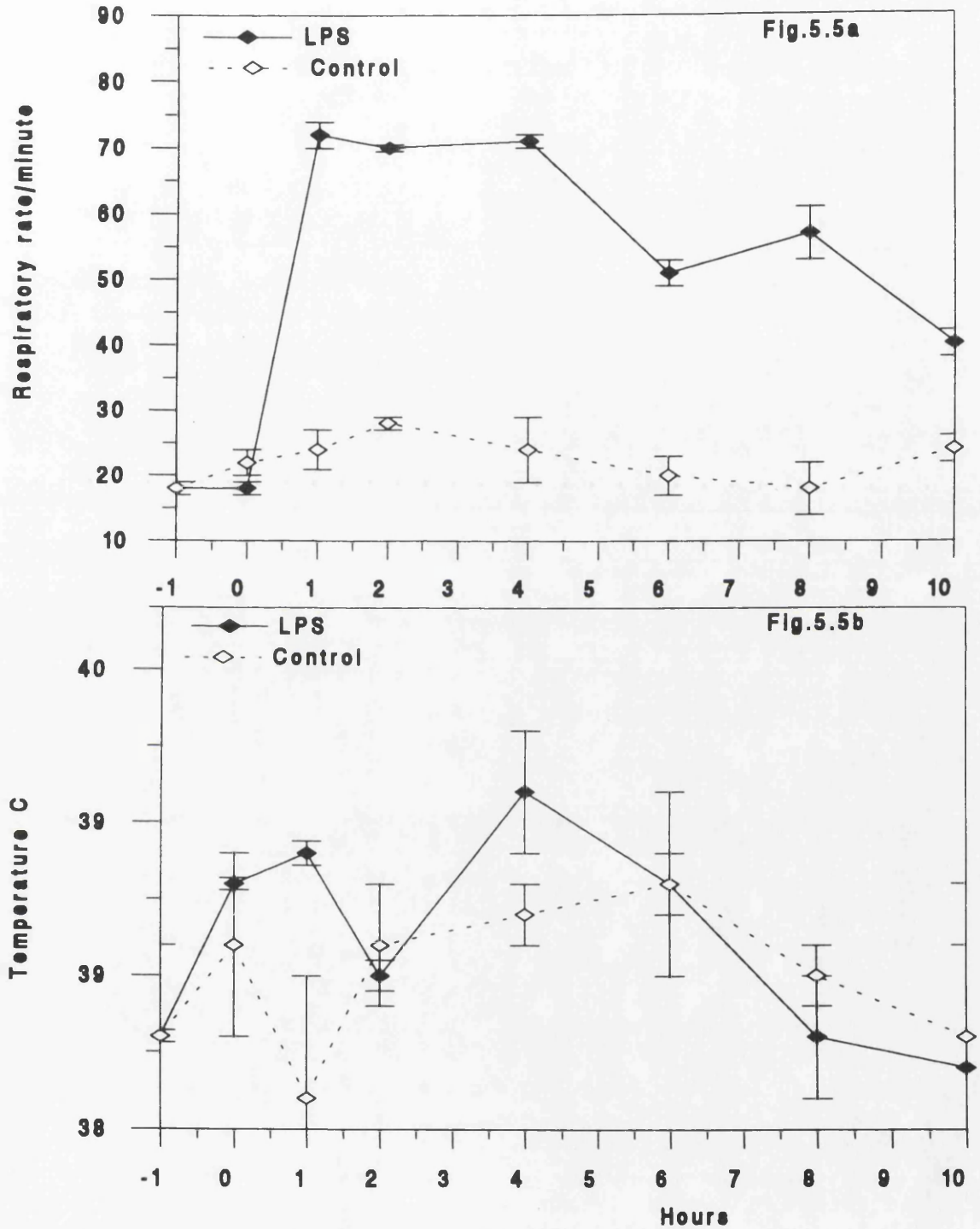


Figure 5.5 Mean (± SEM) respiratory rates (Fig. 5.5a) and rectal temperatures (Fig. 5.5b) of calves over 10 hours after intravenous administration of *P. haemolytica* LPS (0.7 μg kg^{-1} in 10 ml PBS; n=5). The control calves received 10 ml phosphate buffered saline intra-tracheally (n=5).

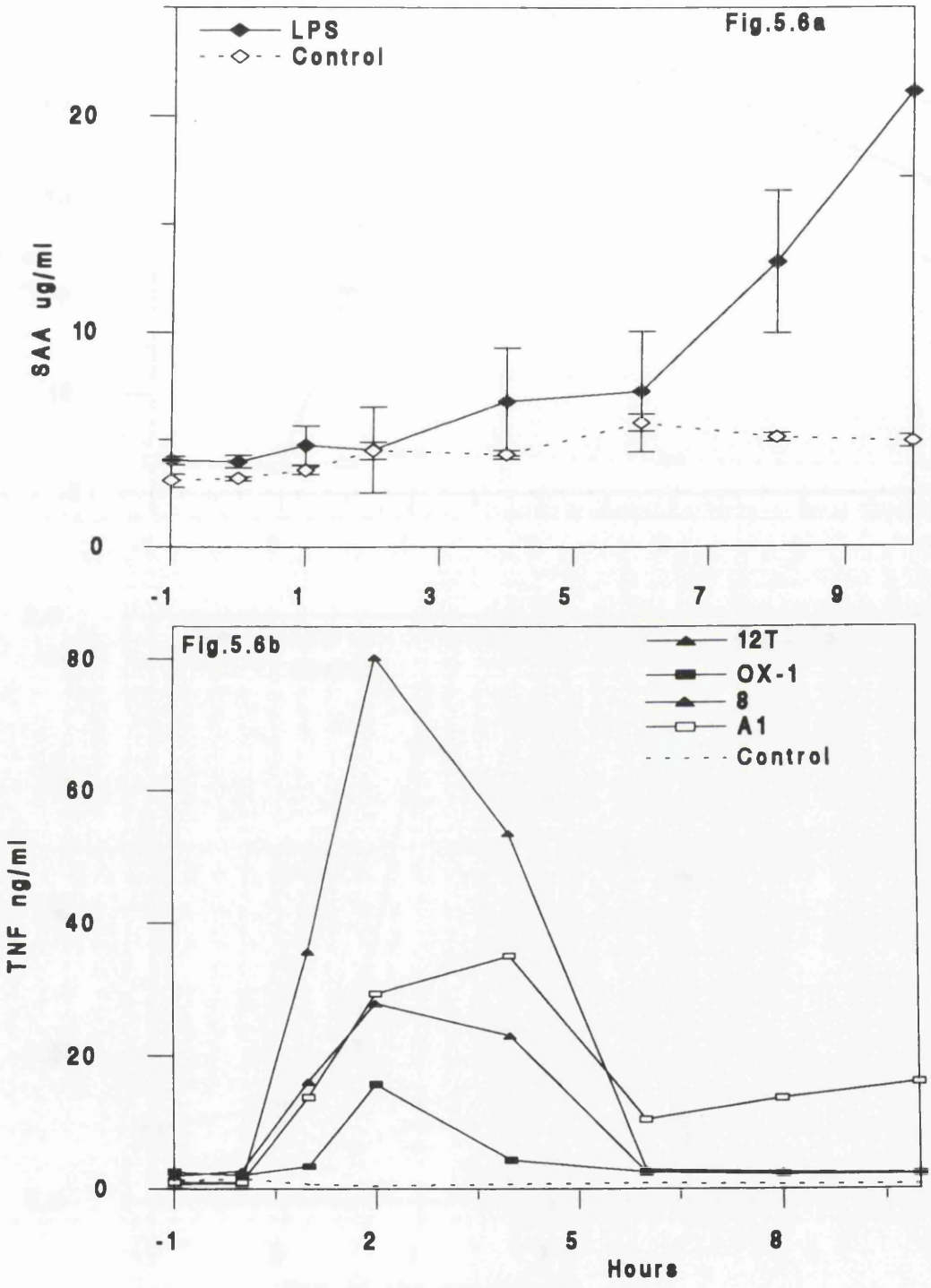


Figure 5.6 Mean (± SEM) serum amyloid A concentrations (Fig. 5.6a) and the individual tumour necrosis factor$_{\alpha}$ concentrations (Fig. 5.6b) after intravenous administration of *P. haemolytica* LPS (0.7 μg kg^{-1} in10 ml PBS. n=3); animal No. A1 received 1.0 μg kg^{-1} *P. haemolytica* LPS. The control calves were given 10 ml PBS, intravenously.

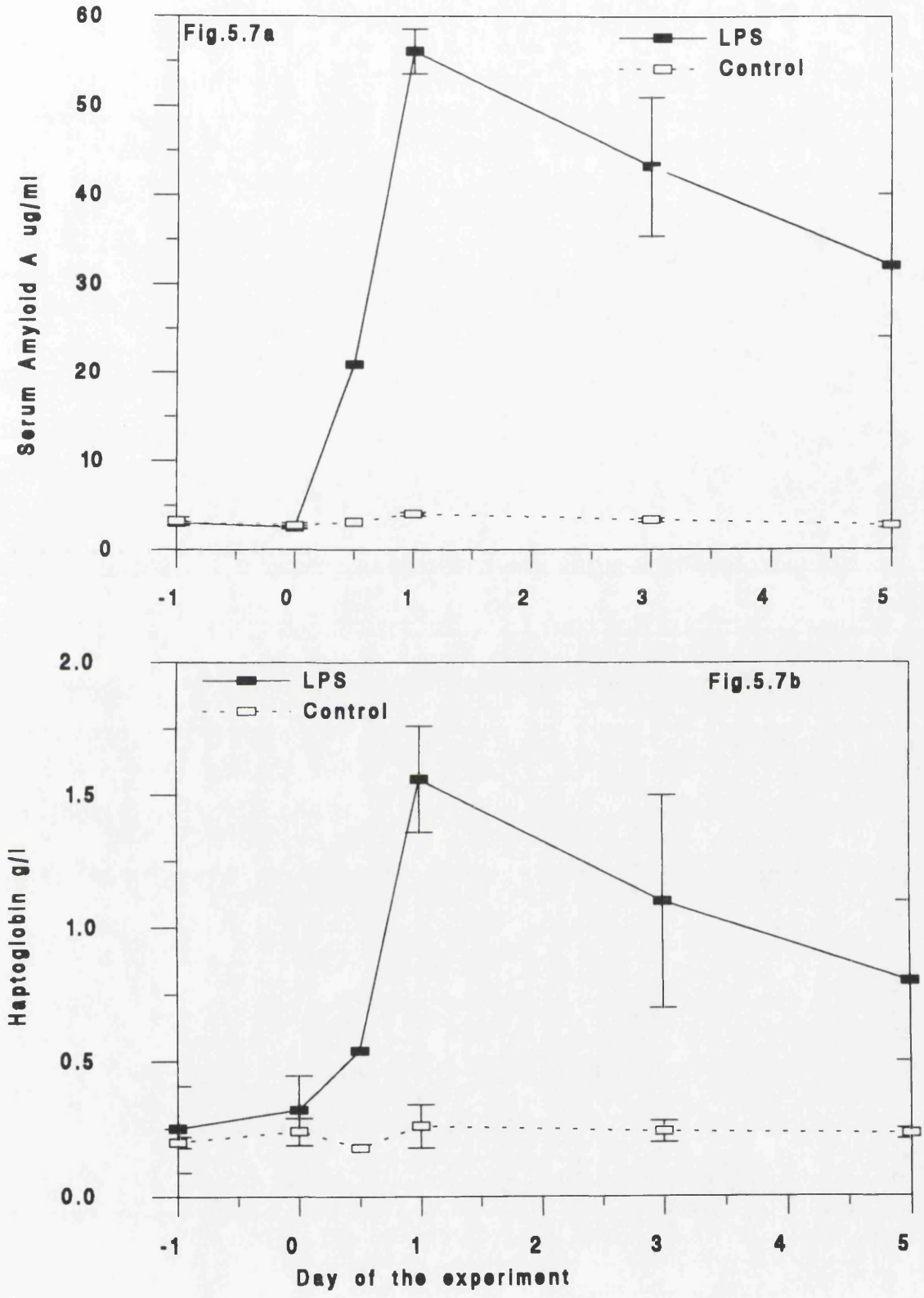


Figure 5.7 Mean (± SEM) serum amyloid A concentrations (Fig. 5.7a) and the haptoglobin concentrations (Fig. 5.7b) in calves over 5 days after intravenous administration of *P. haemolytica* LPS (n=5). The control calves were given 10 ml PBS, intravenously.

1.5 times the control value on day 3 and further decline was found on day 5 post-treatment (Figure 5.8a).

5.3.3.2. Haematology

In the haematological studies, the main changes observed were in the total leukocyte and neutrophil counts (Fig 5.8b). Before inoculation the total leukocyte counts in both groups were similar and within the reference range. Following LPS infusion there was a marked increase ($P<0.001$) in total leukocyte counts in the endotoxin-treated group which reached a maximum after 24 hours while in the control group leukocyte counts remained unchanged throughout the entire study (10.6 ± 1.0 to $10.8 \pm 1.3 \times 10^9\ l^{-1}$). The leukocytosis reflected an increase in neutrophil counts, (from 2.8 ± 0.4 at pre-treatment to $11.3 \pm 1.8 \times 10^9\ l^{-1}$; after 24 hours; $P<0.001$). Monocyte counts gradually reduced after LPS injection from 0.8 ± 0.03 to $0.45 \pm 0.01 \times 10^9\ l^{-1}$ after 6 hours and started to rise gradually reaching $0.75 \pm 0.23 \times 10^9\ l^{-1}$ after 24 hours but these were not significant changes. LPS also induced a decrease in lymphocyte counts from 7.3 ± 0.25 at the time of injection to $6.2 \pm 0.3 \times 10^9\ l^{-1}$ after 6 hours with a recovery to 8.3 ± 0.4 after 36 hours but again these were not significant changes (Fig.5.8b.) .

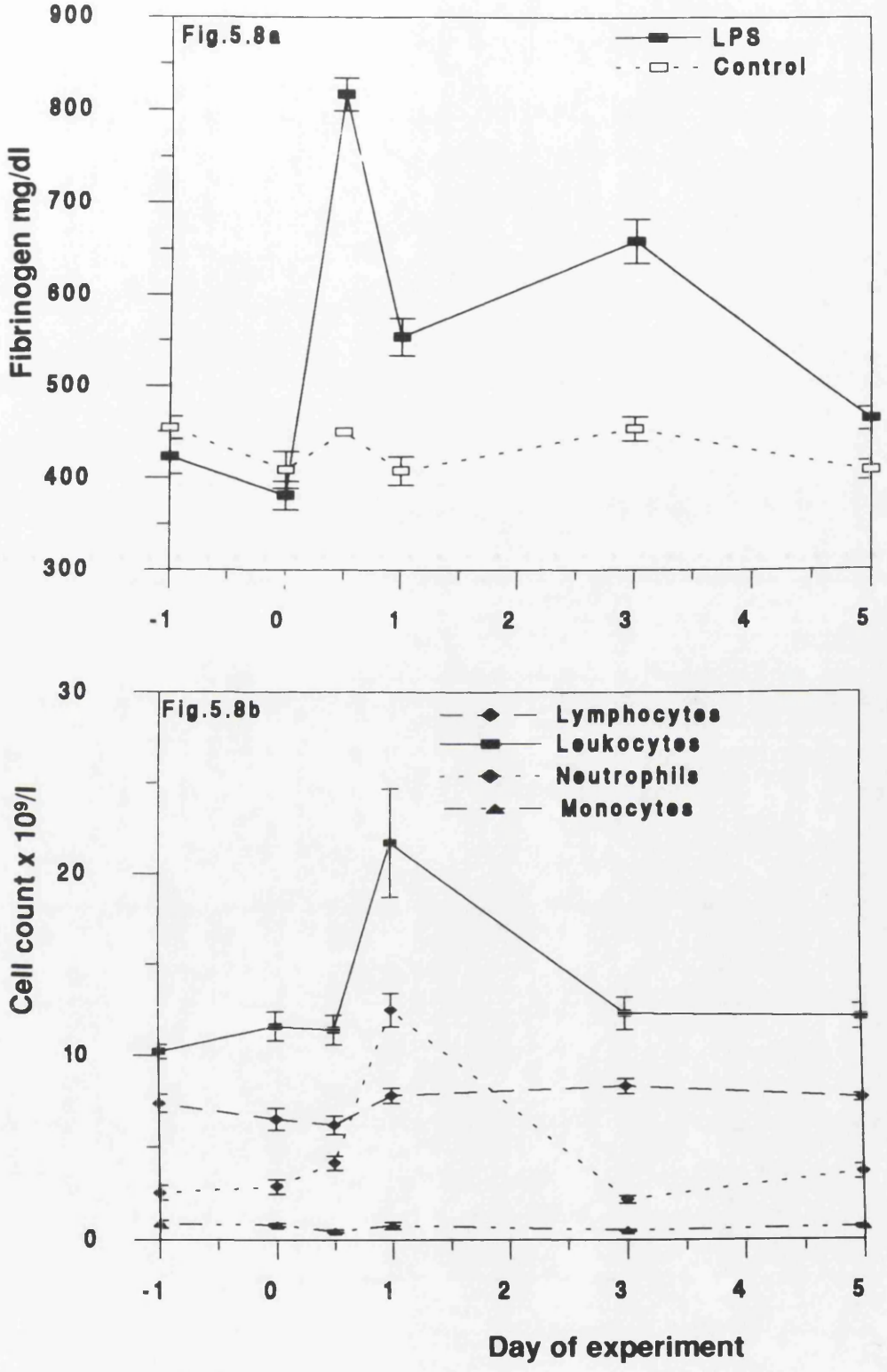


Figure 5.8 Mean (± SEM) serum fibrinogen concentrations (Fig. 5.8a) in calves over 5 days after intravenous administration of *P. haemolytica* LPS. The control calves received phosphate buffered saline intravenously (10 ml per calf; n=5;). Figure 5.8b shows the mean (± SEM) total leukocyte, neutrophil, lymphocyte and monocyte counts in calves over a 5 day period after intravenous administration of *P. haemolytica* LPS.

5. 4. Discussion

The main objectives of this study were to determine the involvement of the cytokine network in the immediate APR in bovine pasteurellosis and to identify what role, if any, is played by LPS from *P.haemolytica* in the initiation of the systemic reaction to this infection. In these experiments blood samples were collected more frequently than in the studies described in Chapter 4 and were therefore more suitable to extending our knowledge of the onset of the APR in cattle.

The results of this study demonstrates that both *P. haemolytica* and LPS extracted from the bacteria stimulate an APR within a few hours of administration. The presence of a systemic APR was confirmed by the clinical changes which included a significant increase in respiratory rate (>60 min^{-1}), associated with pyrexia and anorexia. These findings agreed closely with the observations reported by Gibbs *et al.* (1984) and demonstrated that the alterations produced during the APR to *P. haemolytica* could be instigated by LPS *via* the cytokine net work.

Previous studies by Conner *et al.* (1989) and Horadagoda *et al.* (1992) on BPP have demonstrated that an acute phase protein response is detectable 24 hours after infection with *P.haemolytica.* This is the first study to observe the immediate acute phase protein response in BPP and to demonstrate a progressive increase in SAA concentrations during the first 10 hour period when Hp concentrations remained small and insignificant.

It is accepted that in man and other laboratory animals, the APR synthesis seen during inflammation is regulated by several cytokines, particularly IL-1, IL-6 and TNF_{α} (Evans and Whicher 1992; Koj 1985). However, controversy exists regarding the quantitative and qualitative importance of these three cytokines. For example in human hepatocytes, SAA mRNA is increased modestly by IL-1

while it remains constant or is decreased by IL-6. In contrast, IL-6 induces fibrinogen mRNA in a dose dependent manner whereas IL-1 reduces this effect (Malawista *et al.* 1989; Moldawer *et al.* 1989; Playfair 1991).

The frequent sampling protocol adopted in the present study demonstrated a rise in circulating SAA as early as 4 hours after infection but Hp did not increase above the normal concentration during the 10 hours following infection. A possible explanation for the difference in the SAA and Hp responses observed in this study may be due to the difference in the induction of hepatic synthesis which may be related to sequential release of cytokines. Haptoglobin is a glycoprotein and its production is dependent on IL-6, the cytokine primarily responsible for regulation of APP production by hepatocytes (Koj 1985). However, SAA is an apolipoprotein and IL-6 is not the main mediator of SAA production (Playfair 1991; Hocke *et al.* 1992). Early investigations identified IL-1 as the main inducer of SAA (Sipe *et al.* 1979; Vogal and Hogan 1990; Playfair 1991) but more recent *in vitro* and *in vivo* studies have demonstrated that IL-1 is not the only cytokine that modulates acute phase SAA synthesis and have implicated TNF_{α} as a major mediator of SAA (Sipe *et al.* 1987; Sipe 1990).

The question of whether the earlier stimulation of SAA production was a result of the sequential and differential stimulation by cytokines could be addressed by characterisation of the sequence of the cytokine response to infection. This was a further stimulus to provide suitable assay systems for the bovine cytokines IL-1, IL-6 and TNF_{α}. However, despite the unsuccessful attempts to measure b-IL-1 and b-IL-6, the availability of an RIA for b-TNF_{α} has demonstrated an early TNF_{α} response following post-infection which is in agreement with similar studies conducted in other species (Waage *et al.* 1987; Michie *et al.* 1988).

The use of the specific radioimmunoassay for bovine TNF_{α} to analyse the serum samples taken after *P. haemolytica* inoculation demonstrated that a TNF_{α} response was induced as early as 1 hour after the inoculation and a peak concentration of 18 - 37 ng ml^{-1} was achieved within 2 hours of inoculation with non-detectable levels restored by 6 hours. The rapid rise and fall in serum TNF_{α} is compatible with the short half-life of serum TNF_{α} reported for various species, for example 72-90 minutes in both humans and rats (Michie *et al.* 1988; Waage *et al.* 1992) and 6-7 minutes in mice (Beutler and Cerami, 1989). These results also confirm a recent report of a systemic TNF_{α} response during experimental bovine pasteurellosis (Pace *et al.* 1993). Nevertheless, there are qualitative and quantitative differences between the two studies. Pace *et al.* (1993) identified low and high responding animals, with maximum serum TNF_{α} concentrations in their high responding calves of about 6 ng ml^{-1}. The peak serum concentrations of TNF_{α} reported in the present work were higher (18 to 37 ng ml^{-1}) and occurred 2 hours post-challenge rather than 8 hours post-challenge as reported by Pace *et al.* (1993). These workers also reported a longer measurable TNF_{α} response (>72 hours). Differences between the results presented here and those of Pace *et al.* (1993) may be due to the cytotoxic biological assay which they employed. Biological assays are not specific for TNF_{α} estimation unlike the RIA which was used in this study. Both TNF_{α} and TNF_{β} are toxic to a range of tumour cells *in vitro* and cytotoxic assays are not capable of differentiating these forms of the cytokine (Meager *et al.* 1989). Therefore it is possible that the bioassay used by Pace *et al.* (1993) could have measured TNF_{α} along with TNF_{β} which is known to have a longer half life (Nelson *et al.* 1990).

The results of this investigation also disagreed with those of Espinasse *et al.* (1993) who employed a RIA similar to that described here, based on rb-TNF_{α}, to examine serum following experimental BPP infection. In this investigation the inoculum was administered as a single dose whereas Espinasse *et al.* (1993)

inoculated calves over 3 consecutive days. After the initial burst of TNF_{α} release, the cells such as the alveolar macrophages, producing TNF_{α} become resistant to the stimulating effect of LPS (Waage 1987) and the TNF_{α} production begins to fall. Therefore the inoculation regime of Espinasse *et al.* (1993) may have been too low and too extended to produce a detectable increase in TNF_{α}. Additionally, transient accumulation of TNF_{α} in the serum may reflect an acute over-production followed by a spillover phenomenon when local tissue binding sites have been saturated and or there has been temporary breakdown of natural inhibitors (Tracey *et al.* 1989; Creasy *et al.* 1991).

Another reason for the inability of Espinasse *et al.* (1993) to detect TNF_{α} is likely to be because of the low frequency of bleeding as sampling once or twice daily would not allow a rapid peak to be detected. This combined with the multiple inoculation patterns used is most likely to be the main reason for the difference in detection of the TNF_{α} response to BPP between the investigation described here and that of Espinasse *et al.* (1993). Their assay system which was also based on the RIA of Kenison (1990; 1991) has successfully identified a TNF_{α} response in calves infected intravenously with *E. coli* (Peel *et al.* 1990) but following *S. typhymurium* infection they failed to demonstrate a TNF_{α} peak. Blood samples were again collected only once a day and this could easily cause a TNF_{α} peak in the serum to be over looked (Peel *et al.* 1990).

Unfortunately, attempts to demonstrate the sequential stimulation of cytokines in BPP were unsuccessful due to the inability of bioassays for human IL-1 and IL-6 to quantify the bovine cytokines in the serum samples. In an earlier study, Winstanley and Eckersall (1992) measured bovine IL-1 like activity in the supernatant from bovine mononuclear cells stimulated with LPS and demonstrated that the D-10 cell line (Winstanley and Eckersall 1992) which has been used to measure human IL-1 could be used to measure bovine IL-1 in culture medium. However, in preliminary studies D-10 cells failed to proliferate with the bovine serum samples from animals infected with *P.haemolytica.* Therefore, an attempt

was made to measure bovine IL-1 like activity using an alternative method described by Collins *et al.* (1989). This assay was originally developed to measure IL-1 activity in bovine macrophages collected from injured mammary glands and used a bovine thymocyte cell culture as the bioassay system.

However, the assay of serum IL-1 activity in BPP using the calf thymocyte cell line was not successful and this could be attributed to at least two reasons. The first and most likely reason is that the IL-1 response in pasteurellosis was too low for detection by this assay even though it had a limit of detection of 0.1 pg ml^{-1} of IL-1. The second possibility is that IL-1 inhibitors or binding protiens present in the bovine serum may have inhibited a reaction in the IL-1 bioassay. Such inhibitors have been identified in blood and other biological fluids (Larrick 1989). This possibility was tested by adding rb-IL-1 to serum from infected animals which resulted in no loss of activity of the cytokine added to the serum. Therefore the possibility of an inhibitory effect of bovine serum on the thymocyte response could be virtually eliminated. Various methods have been used to remove potential inhibitors of IL-1, including serial dilutions, chloroform extraction (Cannon *et al.* 1988) and treatment with PEG (Hopkins *et al.* 1990). These procedures however, were not attempted in the present assay of bovine IL-1 but could be recommended for any further attempts to estimate IL-1 by bioassay. To confirm the production of IL-1 in BPP, alveolar macrophages obtained through bronchoalveolar larvage (BAL) may be a better source of material as these cells are likely to have a higher concentration of this cytokine and effects of serum inhibitors would be virtually eliminated. However, the dilution effect of lavage buffer would reduce the quantitative value of such an approach. Another alternative is to use peripheral blood lymphocyte's (PBL's) which may be a better source than the serum. It is likely that detection of bovine IL-1 in serum from infected animals will have to wait until an ELISA or a radioimmunoassay system has been developed for the bovine cytokine.

Although IL-1 like activity was not demonstrated in the serum of infected animals, clinical features such as fever, anorexia, pain and lethargy which are associated with IL-1 in other species (Dinarello 1984b; Atkins 1989) were observed in the infected calves, suggesting a major role for IL-1 in mediating the APR in bovine pasteurellosis.

In the present study the attempt to measure circulating bovine IL-6 like activity using a bioassay was not successful. As in the case of the attempt to measure bovine IL-1 by bioassay, this may be either because the assay was not sensitive enough or due to the interference of bovine serum with the bioassay.

Bovine serum from animals before and after experimental infection with *P.haemolytica* reduced the uptake of [^{3}H]-thymidine in the B9 cell line below that of the negative control, indicating that cells were being inhibited rather than being stimulated. This was in contrast to the IL-1 assay, where bovine serum had no effect on the basal metabolism of the bovine thymocytes. The effect on the IL-6 bioassay may to be due to a toxic effect of the calf serum but was not related to the infection as pre-infection serum had the same effect. The toxic effect decreased as the serum samples were titred out. This assay is in regular use for the measurement of feline IL-6 in purified peripheral blood lymphocyte's (PBL's), serum and plasma from cats with feline infectious peritonitis (FIP) and this toxic effect has not been seen with any feline sera present at the same range of dilutions (Dr. C. Lawrence, personal communication). Whether the IL-6 assay is sensitive enough for analysis of bovine serum will have to wait until the problem of the toxic effect of the serum is solved. Foetal bovine serum (10% v/v) was one of the constituents of the tissue culture media and therefore, FBS must not contain the component with the toxic effect on the cell line, whereas bovine serum from the 3 months old calves used in the experiment appears to contain inhibitory factors. One possibility is foetal bovine serum is subjected to processing prior to sale which would destroy or remove the toxic factor. Initial attempts to remove the

inhibition by heat treatment (56°C for 30 min) and the addition of PEG to the serum did not alter the B9 cell line response to bovine serum.

Studies in cattle have revealed that an acute phase protein response was observed after infusion of human-recombinant-IL-6 (Nakajima *et al.* 1993) . The serum concentration of Hp and fibrinogen responded to rh-IL-6 by increasing 100-fold and doubling respectively. In the present studies (see Chapter 4) both SAA and Hp were raised in response to *P. haemolytica* infection and to treatment with LPS which suggests that IL-6 like activity is a likely mediator of the APR in bovine pneumonic pasteurellosis, but confirmation of this postulate will require a suitable assay for measuring IL-6 either by overcoming the toxic effect on the B9 cell line, by development of alternative cell lines not susceptible the toxic effect or by development of an immunoassay.

Investigation of the interaction between bacteria and host showed that a distinct acute phase response was found after treatment of calves with LPS even though LPS from 4 field isolates and different doses (0.7 and 1.0 $\mu g\ kg^{-1}$ body weight) were used. The response was evident from the clinical signs, the acute phase protein response, the haematological response and by demonstration of a TNF_{α} response within 2 hours of infection. Indeed, few of the systemic acute phase responses which occur in BPP were not mimicked by LPS treatment. Unfortunately it was not feasible to measure all the analytes studied in Chapter IV for their responses to LPS. It would be of particular interest to extend the investigation to cover hormone and trace element responses. During the immediate response, TNF_{α}, Hp and SAA gave similar responses to LPS as that seen in *P.haemolytica* (Experiment I). However, rectal temperature did not show a significant rise with LPS but the respiratory rate was much sharper with the LPS response. When the acute phase response to LPS was examined over 5 days a similar response to that obtained with the whole bacterium was found and there was little difference in the course of the response for these analytes. There were differences in the extent of the response in comparison to that shown by

P. haemolytica infection (Experiment I), but these may only relate to the doses of LPS used in the experiment and further investigation with increasing doses of LPS are required to clarify this point. However, these initial studies demonstrate that most, if not all, of the systemic effects produced by *P. haemolytica* on the metabolism of the host can be caused by LPS and its interaction with leukocytes and the cytokine net work. It would be of interest to determine whether the systemic response is influenced more by LPS transferring from the site of infection in the lung to the circulation or by activation of alveolar macrophages and cytokine from the latter causing the systemic response. Evidence from this study indicates that LPS from *P. haemolytica* would be able to induce a rapid APR once it is in the circulation. Investigations to measure the serum LPS level in the early APR would help to answer this question.

Experiments carried out in mice indicate that in LPS-induced inflammation, the TNF_{α} concentration in serum increased from 15 minutes after LPS administration, reached a peak 85 minutes later and was not detectable 200 minutes (Remick *et al.* 1987) after administration. There is strong evidence that the generation of TNF_{α} is a principal mediator of the APR observed *in vivo* following the administration of LPS. Recombinant TNF_{α} given to rats by intravenous infusion over 5 minutes at a dose of 3.6 mg kg^{-1} causes hypotension, metabolic acidosis, hyperglycaemia, hyperkalaemia and death. However, lower doses of TNF_{α} are compatible with survival (Tracey *et al.* 1986). Monoclonal antibodies to TNF_{α} are protective against potentially lethal doses of endotoxin indicating the important role of this cytokine (Bagby *et al.* 1990). However, such antibody treatment does not abolish all the inflammatory changes seen in LPS-treated animals.

In the LPS experiment the time course of serum TNF_{α} activity after intravenous LPS administration was similar to that studied in other species (mice, chimpanzees) where a peak serum concentration occurring within 90 minutes of infusion and then returned to pre-infected levels 3-4 hr later (Morris 1992).

However, in horses TNF_{α} response time has been shown to be dependent on the method of stimulation (May *et al.* 1992). When animals were treated with a high concentration of LPS from *E coli* (1 mg kg^{-1}) there was a rapid increase in serum TNF_{α} concentration with a rise seen as early as 2 hours post-treatment. However, when smaller doses of LPS (30 ng ml^{-1}) were administered by the same route, a serum TNF_{α} increase was detected only after 3 hours.

Lipopolysaccharide treatment of calves not only caused a peak of TNF_{α} and initiated production of SAA and Hp, but also effected leukocyte concentration in circulation as did inoculation with the whole bacterium (Chapter IV). LPS induced a pronounced neutrophilic leukocytosis in all calves within 24 hours. Similar results have been reported by Bowersock *et al.* (1990) and Breider *et al.* (1990; 1991) in calves exposed to a mixture of LPS and crude leukotoxin of *P. haemolytica.* It is likely, that the concurrent increase in the circulating levels of cytokines contributed to this response because intravenous administration of either TNF_{α}, IL-1 or IL-6 gives rise to neutrophilia in several species (Ulich *et al.* 1987; van Deventer *et al.* 1991). IL-1 in particular, caused great peripheral neutrophilia and lymphopenia. The peripheral neutrophilia was characterised by an increase in circulating band forms (Ulich 1992). In this experiment a slight neutrophil rise was observed as early as 6 hours post-infection.

The migration of neutrophils from the pulmonary microvascular compartment into the pulmonary interstitium and alveolar space is an important immunopathological consequence, since this process is also accompanied by neutrophil activation. In the setting of enhanced vasodilatation and vascular permeability associated with the initial local APR, neutrophils must first adhere to endothelial cells, followed by diapedesis along established chemotactic gradients (Maheswaran 1993; Walker *et al.* 1985). Cellular communication *via* cytokine networks operating between immune and non-immune cells of the alveolar-capillary membrane may be essential for the generation of inflammation

(Maheswaran 1993; Slocombe *et al.* 1990). The subsequent events of these cellular-humoral interactions are pivotal to the initiation and propagation of the inflammatory response leading to pulmonary injury. Both TNF_{α} and IL-1 are necessary for the initiation of acute inflammation but whether TNF_{α} or IL-1 participates in a pulmonary immune reaction is still unclear.

The use of LPS infusion in human volunteers has provided important evidence of the cytokine net work and the associated systemic response (Michie *et al.* 1988). This model, has provided relevant information concerning the cytokine cascade in the human, which has helped to interpret the complex data obtained from infected patients. Similarly studies of LPS treatment of calves will help to identify those biochemical changes dependent on LPS from the bacterium. In cattle, both intravenous administration of LPS and intra-tracheal inoculation of the bacteria induced the acute phase response. This supports the conclusion that LPS is an important mediator in the systemic response to bovine pasteurellosis.

Increased knowledge of the cytokine cascade in man and other species has progressed to the point where therapeutic measures aimed at reducing mortality from bacterial infection by influencing cytokine action have been undertaken (Hannum *et al.* 1990; Silva *et al.* 1990; Eisenberg *et al.* 1991). Low concentrations of cytokines or their receptor antagonists have an important protective effects in host resistance (Havell 1989; Carter *et al.* 1990; Piguet *et al.* 1990) so that, with any anti-cytokine therapy, it will be important to avoid potentially harmful immunosuppressive effects. More research based on antibodies raised against bovine TNFs, interleukins and LPS from bovine pathogens would be beneficial to increase understanding of this complex network of interacting mediators.

In conclusion, this experiment has demonstrated that *P.haemolytica* infection and its LPS induces TNF_{α} *in vivo* probably by activation of bovine macrophages and monocytes. The acute phase response that developed is likely

to be mediated by cytokines including TNF_{α}. Tumour necrosis factor activates the hepatocytes to release APP either directly or *via* other members of the cytokine net work. Unfortunately suitable assay systems were not available to measure interleukins in the present study but the systemic reaction indicates the likely presence of other interleukins. This will be further elucidated when assays become available to detect bovine IL-6, IL-1 and other cytokines.

CHAPTER VI

CONCLUSIONS
AND
GENERAL DISCUSSION

6.1 Conclusions

Serum amyloid A (SAA) was the most responsive of the bovine acute phase proteins in an established animal experimental model for bovine pneumonic pasteurellosis.

Hydrophobic interaction chromatography and gel filtration were the method of choice to purify bovine serum amyloid A (b-SAA) which was used subsequently to quantify a pool of acute phase bovine serum as standard in an enzyme linked-immunosorbent assay to measure SAA in bovine serum.

Infection of calves with *P. haemolytica* A1 caused a rapid fall in circulating zinc and non-haem iron, an immediate rise in cortisol but a fall in thyroxine concentrations, a marked neutrophilic leukocytosis, an increase in bilirubin concentrations and a fall in glutamate dehydrogenase activity.

The response of most of the acute phase reactants related to the extent of the pathological lesions which varied between the two field isolates used to inoculate calves.

P. haemolytica A1 caused a transitory rise in circulating TNF_{α} which was also produced following injection of calves with lipopolysaccharide (LPS) from the bacteria.

Intravenous administration in calves of LPS from *P. haemolytica* A1 caused an acute phase response closely resembling that induced by intra-tracheal inoculation with the whole bacterium.

6.2. General Discussion

Studies described in this thesis and the investigations reported by Boosman *et al.* (1989) have demonstrated clearly that SAA is a major acute phase protein in cattle as it is in man and the horse (Pepys *et al.* 1989; Taktak and Lee 1991). At present SAA concentrations in man are used in clinical monitoring of inflammation and acute disorders such as myocardial infarctions and renal allografts (Yamada *et al.* 1993). Limited studies in domestic animals have also

reported that SAA could be a reliable marker of inflammation and tissue injury in other species (Pepys *et al.* 1989; Boosman *et al.* 1989; Gruys *et al.* 1993). Despite the wide usage of SAA as an acute phase reactant, the precise function of this protein remains unclear. Some workers have implicated SAA in reverse cholesterol transport and more recent studies have suggested that SAA plays a role in the transport of bacterial endotoxins. The availability of purified bovine-SAA offers an excellent opportunity to investigate the specific function of SAA in cattle either by *in vitro* or *in vivo* studies.

At present there are no reports on the structure of b-SAA and, although several isoforms have been described in other species (Steinkasserer *et al.* 1990; Raynes and McAdam 1991) only one isoform of b-SAA has been described (Rossevatn *et al.* 1992). The purification of b-SAA using the methods described in this thesis creates opportunities to investigate the presence of isoforms and to determine the structure of b-SAA using advanced techniques such as crystallography. Additionally, it is now possible to determine the amino acid sequence of the purified protein and compare the homology of b-SAA with SAA from other species.

The b-SAA ELISA developed in this study is the first assay in which the purified analyte has been used to quantify the SAA concentration in SI units. Previous assays for estimating SAA in domestic animals expressed the concentration of SAA in arbitrary units (Boosman *et al.* 1989; Pepys *et al.* 1989). The ELISA described here has a number of other advantages over the ELISA developed by Boosman *et al.* (1989) to measure b-SAA. First the rabbit antibody to bovine amyloid A used by Boosman *et al.* (1989) is not specific to b-SAA and an additional absorption step is necessary before the assay of b-SAA can be performed. Prior absorption of antiserum is not required in the present assay as the rabbit anti-human antiserum reacted specifically with b-SAA in APbS on Western blots. In addition, the SAA antibody used in the present assay is commercially available making the assay widely available and

allowing the assay to be transferable to other laboratories:transfer would be much more limited with the antibody prepared and used by Boosman *et al.* (1989). Furthermore, the use of a calibrated acute phase bovine serum as standard for the assay has overcome the problems associated with the matrix and also reduced the demand for a constant supply of purified analyte to perform the assay.

The analysis of serum from calves experimentally infected with *P. haemolytica* A1 (Chapters IV and V) or inoculated with LPS (Chapter V) demonstrated that SAA is a major acute phase protein in cattle comparable to and complementary with the assay for haptoglobin. In experiments performed to compare the acute phase response (APR) to isolates of *P. haemolytica* (Chapter IV), the SAA concentration reflected the magnitude of the APR observed while the haptoglobin concentrations failed to demonstrate such a difference. Furthermore, in the experiments which examined the immediate acute phase response to *P. haemolytica* infection (Chapter V) the SAA response was present as early as 4 hours post-infection while haptoglobin failed to respond within 10 hours of infection.

The samples from clinical cases analysed for SAA in this study and those performed by Gruys *et al.* (1993) indicate that SAA measurements are reliable in detecting inflammatory conditions in cattle. Furthermore, the greater incremental range of SAA makes it a more sensitive assay when compared to haptoglobin to detect tissue injury caused by infection or trauma. Another application for measurement of SAA as an acute phase protein is as an antemortem test to identify animals with infections or inflammatory diseases which can be then examined further to assess whether the meat from such animals is fit for human consumption.

However, the use of SAA measurements in clinical practice or meat inspection will require a robust, rapid, inexpensive and efficient assay. The ELISA described here, in its present state, does not meet all of these criteria but attempts could be made to improve the assay in the future. Several options are

available for this purpose and include developing a latex agglutination nephelometric immunoassay (Yamada *et al.* 1993) or sequence-specific monoclonal antibodies in an ELISA as described for human SAA (Casl and Grubb 1993).

In the experiments described in Chapter IV a bovine pneumonic pasteurellosis (BPP) experimental model was used to compare the APR of calves to two highly virulent field isolates of *P. haemolytica.* The isolates used for the infection demonstrated similarities in their LPS content but varied in their capacity to produce leukotoxin *in vitro* (Dr. R.L. Davis personal communication). Following inoculation, both isolates demonstrated many of the changes associated with the APR but there was a difference in the magnitude of the response produced by each isolate. Clinically the animals in Group I, which were inoculated with *P. haemolytica* isolate SB/82/1, demonstrated a more severe response in relation to demeanour, rectal temperature, respiratory rate, and food intake compared to animals in Group II inoculated with isolate SC/82/1. Other acute phase responses such as leukocytosis, mineral redistribution, acute phase protein response and endocrine changes were also more prominent in the animals in Group I compared to those in Group II. The findings of these experiments indicate that the APR can be used as a means of studying or comparing the *in vivo* responses of calves to different bacterial isolates without having to resort to post-mortem examination.

The findings of the present study demonstrate for the first time the presence of a trace mineral redistribution during the APR to *P. haemolytica* infection in cattle. The experiments demonstrated a 70 per cent reduction in the iron concentration and a 60 per cent reduction of the zinc concentration within 24 hours of challenge. The redistribution of trace minerals by the host during the APR may play an important protective role by curtailing the multiplication of bacteria by depriving the microorganisms of essential trace minerals which are required for its survival and proliferation. Furthermore, the studies by

Slocombe *et al.* (1990) suggests that the reduction of circulatory iron may decrease the leukocyte-derived toxic metabolites which apparently play a significant role in the initiation of the pulmonary lesions in BPP.

Only a few studies have examined the implication of endocrine changes with infection in domestic animals (Elsasser 1992). Indeed, the investigations reported in this thesis are among the first attempts made to study the endocrine changes associated with the APR in BPP. These studies demonstrated a significant rise in serum cortisol and a marked decline in thyroxine levels during the APR of infected animals. The mechanisms responsible for the hormonal changes are a subject of much debate. However, several *in vitro* and *in vivo* studies performed in laboratory animals have demonstrated a major role for cytokines, particularly IL-1, in direct and indirect stimulation of the adrenal cortex to release cortisol (Besadovsky *et al.* 1986; Salas *et al.* 1990a; 1990b). The specific cause for the reduction of thyroxine is unclear.

Chapter V describes experiments performed to determine the role of cytokines in the systemic response of bovine pneumonic pasteurellosis. As LPS of *P. haemolytica* is recognised as an important virulence factor, animals in one group were infused with LPS extracted from *P. haemolytica* while another group was infected with the whole live organism. Animals in both groups developed an APR which was characterised by pyrexia, anorexia, tachypnoea and an acute phase protein response. Furthermore, the infected animals and those infused with LPS demonstrated a rise in serum TNF_{α} as early as 1 hour after inoculation and a peak at 2 hours with no detectable levels by 6 hours.

The ability of LPS to generate an APR and a TNF_{α} response in the present study confirmed previous investigations in which LPS has been incriminated as a major virulence factor of *P. haemolytica* (Confer *et al.* 1990; Whiteley *et al.* 1992). *In vitro* and *in vivo* studies performed in laboratory animals and man have clearly demonstrated that LPS activates tissue macrophages and monocytes to synthesise and release a wide range of substances

including the cytokines TNF_{α}, IL-1 and IL-6 (Sipe 1990). Furthermore, these studies have shown that TNF_{α} is a key mediator of the APR which is characterised by many pathophysiological alterations. Indeed, in man LPS is known to induce the sepsis syndrome (septic shock) in Gram-negative infections through TNF_{α} stimulation, which can result in multi organ failure and even death (Bone 1991). The results of the present study demonstrate a TNF_{α} response which suggests that this cytokine may play a pivotal role in mediating the APR in BPP following *P. haemolytica* infection and after stimulation by LPS.

The precise origin of circulatory TNF_{α} in the APR following *P.haemolytica* or LPS stimulation is unclear. Whiteley *et al.* (1990) using immunohistochemical techniques, demonstrated the presence of *P. haemolytica* LPS in the intra-vascular, interstitial and alveolar macrophages (AMO) following intra-tracheal inoculation of the organism. These findings suggest that the LPS in BPP could cross the alveolar wall to stimulate pulmonary intravascular macrophages (PIM) in addition to the AMOs to produce TNF_{α}. This postulation however, needs to be confirmed by demonstrating circulatory LPS in BPP. Studies conducted in the rats have also shown that the LPS administered intra-tracheally crosses the alveolar wall to disseminate into visceral organs (Freudeberg *et al.* 1984). In contrast, investigations in sheep using radio-labelled *P. haemolytica* LPS have noted that the LPS does not cross the alveolar wall and was restricted to the alveolar macrophages, suggesting that alveolar macrophages are the main source of TNF_{α}. The active role of alveolar macrophages in restricting LPS may partly explain the reason for the inability to experimentally establish pneumonic pasteurellosis in conventional sheep (Personal communication Dr. J C.Hodgson).

The demonstration of TNF_{α} in the APR of BPP have implications on studies related to the pathogenesis of the disease and suggest the need for investigations on the use of therapeutic alternatives such as the use of low doses of recombinant-TNF_{α}, IL-1 receptor antagonist, anti-TNF_{α} and anti-LPS which

are being actively studied and used in human medicine to minimise the deleterious effects of LPS in Gram-negative infections (Wong and Goeddel 1988; Carter *et al.* 1990; Waage *et al.* 1992). However, at present this may not be a feasible proposition under field conditions, but with the development of sequence-specific monoclonal antibodies it may be a therapeutic approach worth considering in the future.

Experimental studies in other species have shown that clinical signs such as fever, anorexia, pain and lethargy in the APR are associated with IL-1 activity (Dinarello 1984a; 1984b). The presence of comparable clinical signs during the APR in BPP suggests a role for IL-1 although the bioassays used in this study failed to demonstrate the presence of this cytokine. One reason for not detecting IL-1 activity may be the low serum concentration in BPP which was perhaps below the assay detection limit. IL-1 is produced by numerous cells therefore future attempts may be made to circumvent the problem associated with the low serum concentrations by the use of an alternative source for IL-1 measurement such as peripheral blood lymphocytes or alveolar macrophages obtained by bronchoalveolar lavage. Furthermore the use of cells as a source for IL-1 estimation will overcome the effect of IL-1 inhibitors in plasma such as corticosteriods, α-melanocyte stimulating hormone and PGE_2 which can interfere in bioassays (Larrick *et al.* 1989). The measurement of IL-1 activity in serum would be more convenient, but this may have to wait till the development of a suitable ELISA or RIA.

IL-6 is recognised as a potent inducer of acute phase protein response in man and laboratory animals (Gauldie *et al.* 1987; Kishimoto *et al.* 1992), and experiments performed by Nakajima *et al.* (1993) have demonstrated that recombinant human IL-6 has a similar effect in cattle. The results of the acute phase response of BPP investigated in this study have demonstrated an acute phase protein response characterised by an increase of several acute phase proteins including SAA, fibrinogen and haptoglobin which suggest a major role

for IL-6 activity. However, the human and feline IL-6 bioassay used here to measure bovine IL-6 activity failed to demonstrate the presence of this cytokine in serum. These observations indicate the need for development of species specific assays for estimating bovine IL-6 activity in the APR.

In the experiments described in Chapter V the TNF_{α} response was examined after a single inoculation of *P. haemolytica* LPS. Studies in man (Granowitz *et al.* 1993) using a continuous LPS infusion have provided an opportunity to study the sequential release of cytokines in the APR. Such an experimental procedure in the future will provide a clear insight to relative cytokine production which could be correlated with changes present during the APR. The experiments described in this thesis have concentrated on the cytokines responsible for the induction of the APR however, future studies should not overlook the role of cytokines such as IL-4 and IL-8 which are implicated in the down regulation of the APR. At present cytokine research in domestic animals is at an early stage but there are considerable scope for such investigations in order to understand the host responses which are now recognised to have a major role in disease processes.

APPENDICES

Page

LIST OF APPENDICES(Continued)

Appendix 2.1

Stock solutions, SDS-PAGE buffers and gel mixes

Stock Acrylamide

acrylamide	29 g
methylene-bisacrylamide	1 g
dH_2O	100 g

SDS-PAGE resolving gel

stock acrylamide	10.0 ml
1M Tris-HCL (pH8.8)	5.0 ml
dH_2O	4.6 ml
10% SDS	200 µl
10% APS	100 µl
TEMED	10 µl

SDS-PAGE resolving gel

stock acrylamide	2.5 ml
1M Tris-HCL (pH6.8)	2.5 ml
dH_2O	8.0 ml
10% SDS	200 µl
10% APS	100 µl
TEMED	10 µl

SDS-PAGE tank buffer

glycine	14.4 g
Tris base	3.1 g
SDS	1.0 g
dH_2O	1 litre

Treatment buffer (sample buffer)

2.5% SDS
2.5 % dithreioerythriol
0.1 M Tris-HCL (pH6.8)
10% glycerol
0.01% bromophenol blue

Coomasie Blue staining solution

Coomassie blue	0.5g
methanol	300 ml
glacial acetic acid	100 ml
dH_2O	to 1 litre

Destaining solution

methanol	300 ml
glacial acetic acid	100 ml
dH_2O	to 1 litre

Appendix 4.a

Composition of Cortisol radioimmunoassay buffer

Reagents

Sodium dihydrogen orthophosphate $NaH_2PO_4.2H_2O$ 2.18 g l^{-1}

Disodium hydrogen orthophosphate Na_2HPO_4 12.21 g l^{-1}

Sodium azide (NaN_3) 0.50 g l^{-1}

Adjust pH to 7.4

Add 1.0 g of gelatin per litre of assay buffer before use.

Appendix 4.b

Preparation of thyroxine for T_4 assay accuracy

To measure accuracy a thyroxine sample with a known concentration was prepared using L-thyroxine (FW 888.9) sodium salt pentahydrate crystalline (Sigma Chemical Company T 2126). 88.9 mg of thyroxine was weighed out and dissolved in 100 ml of a propylene glycol : distilled water (1:1) solution [A] which was adjusted to pH 9 by dropwise addition of 1 M NaOH. Then 0.4 ml of [A] was added to 4 ml of 0.04 M NaOH in duplicate and 0.4 ml of propylene glycol:distilled water solution was added to 4 ml of 0.04 M NaOH used this to blank the spectrophotometer. Absorbance was measured at 325 nm, the ε for thyroxine is 6,200 the spectrophotometer readings for blank was = 0; [A] = 0.545 and 0.528 in the duplicate samples, from this the concentration of [A] was calculated and was 0.95 mmol l^{-1}.

Solution [A] was further diluted twice as follows 1 ml of [A] in 50 ml of barbitone (pH 8.6) buffer (Sigma) solution [B] which was 19.0 and 1 ml of [B] was again diluted with 20 ml of the same buffer (pH 8.6) to make a solution [C]. The concentration of 30 µl of [C] is 27.4 nmol l^{-1}. Take known amounts (30 µl, 20 µl) of [C] and mix with 1 ml of serum and analyse the thyroxin concentration.

Appendix 4.0

Individual dullness scores of the calves infected with *P. haemolytica* A 1

		Day of the experiment												
		-1	0	0	1	1	2	2	3	3	4	4	5	5
Group	**Calf**	+	+	#	+	#	+	#	+	#	+	#	+	#
	2	0	0	1	2	2	1	2	2	2	2	2	2	2
I	3	0	0	3	2	3	*							
Isolate	8	0	0	2	1	2	2	2	2	2	2	2	2	3
(SB/82/1)	9	0	0	1	2	**								
	14	0	0	2	1	2	1	1	1	1	1	0	0	0
	21	0	0	2	2	1	1	1	1	1	1	1	1	1
	2	0	0	1	3	1	0	1	1	2	1	2	1	2
II	4	0	0	1	1	1	1	1	1	1	1	1	1	1
Isolate	5	0	0	1	2	1	0	1	2	1	1	1	2	2
(SC/82/1)	7	0	0	1	2	1	0	1	2	1	1	1	1	1
	21	0	0	1	1	1	0	1	1	1	1	1	1	1
	24	0	0	1	1	0	0	2	1	1	1	1	1	1

* died ** slaughtered on humane grounds, 0= normal, bright, alert; 1 = slightly dull or depressed, easy to catch and hold; 2 = recumbent on first examination but rose on minimal stimulation; 3 = very dull indeed, refused to rise or did so only after maximal stimulation

Appendix 4.1

Individual rectal temperatures (°C) of calves infected with two isolates of *P. haemolytica* A1

		Day of the experiment												
		-1	0	0	1	1	2	2	3	3	4	4	5	5
Group	**Calf**	+	+	#	+	#	+	#	+	#	+	#	+	#
	2	39.3	39.4	39.4	40.0	41.0	40.6	40.7	40.6	40.0	40.7	40.8	40.8	40.9
I	3	39.1	39.6	40.2	38.0	38.5	*							
Isolate	8	39.3	39.1	39.7	39.6	39.8	40.1	40.1	39.6	40.1	40.4	40.4	39.9	39.8
(SB/82/1)	9	39.3	39.3	39.0	37.5	**								
	14	38.5	38.7	39.2	39.2	38.9	38.8	39.3	38.7	39.3	38.8	39.4	38.8	39.5
	21	39.1	39.0	40.2	40.4	41.0	39.8	40.0	39.6	40.0	39.3	39.6	40.0	39.6
	2	39.1	39.0	39.7	38.8	38.6	38.8	39.4	38.9	39.0	39.1	39.2	38.9	39.2
II	4	39.4	38.8	39.5	38.9	39.3	39.2	38.9	39.1	39.1	38.9	38.8	39.4	39.1
Isolate	5	38.8	39.0	40.4	38.8	38.5	38.8	39.2	38.5	39.3	39.1	39.2	39.2	39.1
(SC/82/1)	7	38.9	38.8	39.9	38.4	38.4	38.6	39.2	38.5	38.6	38.3	38.6	38.6	39.2
	21	38.8	39.0	39.2	39.0	39.0	39.1	39.2	38.6	39.0	38.8	38.8	38.9	39.0
	24	39.1	39.7	39.7	38.9	39.1	38.8	39.0	38.9	38.6	39.1	39.0	38.9	39.1

** died *** slaughtered on humane grounds + time 10.00 hours # 16.00 hours

Appendix 4.2

Individual respiratory rates (min^{-1}) of calves infected with two isolates of *P. haemolytica* A1

		Day of the experiment												
		-1	0	0	1	1	2	2	3	3	4	4	5	5
Group	Calf	+	+	#	+	#	+	#	+	#	+	#	+	#
	2	36	30	66	72	64	66	84	80	78	78	84	72	72
I	3	28	24	72	54	40	*							
Isolate	8	32	24	72	66	64	60	72	60	60	48	48	52	42
(SB/82/1)	9	28	24	72	72	**								
	14	32	20	84	66	60	66	60	52	60	60	66	60	54
	21	32	18	72	84	60	60	60	52	72	60	60	66	60
	2	30	24	78	48	56	48	60	78	78	76	50	64	36
II	4	30	24	78	56	44	54	62	46	46	76	36	46	36
Isolate	5	28	24	60	48	48	54	40	46	76	48	44	40	54
(SC/82/1)	7	28	36	72	90	60	48	58	76	58	38	32	42	48
	21	28	24	72	48	42	48	48	40	48	72	42	44	42
	24	36	30	66	60	76	48	80	68	58	68	64	64	42

* died ** slaughtered on humane grounds + time 10.00 hours # 16.00 hours

Appendix 4.3.1.

Group total food intake

Group	Day of experiment	-1	0	1	2	3	4	5
I	Total Feed Intake (kg)	16.5	0	3	7	6	6.5	6.5
Isolate	Total Calf Body wt.	624	624	433	433	433	433	433
(SB/82/1)	Intake kg/50 kg L.wt.	1.32	0	0.35	0.81	0.69	0.75	0.75
II	Total Feed Intake (kg)	26.0	15.5	25.0	29.5	29.0	25.0	24.0
Isolate	Total Calf Body wt.	810	810	810	810	810	810	810
(SC/82/1)	Intake kg/50 kg L.wt.	1.61	0.96	1.54	1.82	1.79	1.54	1.48

L.wt. live weight Each group consisted of 6 calves. Two calves died on day 1 in Group I

Appendix 4.3.2.

Individual body weights (kg) of the calves infected with *P. haemolytica*A1

Group	Calf	Day of the experiment		
		- 6	0	+6
	2	86	83	70
I	3	92	90	*
Isolate	8	98	97	85
(SB/82/1)	9	105	101	**
	14	147	148	140
	21	110	105	95
	2	125	135	130
II	4	140	150	150
Isolate	5	130	140	130
(SC/82/1)	7	100	100	100
	21	140	140	145
	24	140	145	150

* died ** slaughtered on humane grounds

Appendix 4.4.

Individual WBC and neutrophil counts of calves infected with *P.haemolytica* A1 isolate SC/82/1

		Day of the experiment										
		-1	0	0	1	1	2	2	3	3	4	4
x10⁹ l⁻¹	**Calf**	+	+	#	+	#	+	#	+	#	+	#
WBC	2	10.4	10.8	11.6	21.7	19.4	14.5	14.9	12.5	11.3	11.3	11.3
	4	6.0	5.5	8.5	9.4	7.3	5.9	6.5	6.2	6.5	6.2	6.8
	5	10.0	9.2	11.4	16.4	14.4	9.7	9.9	8.1	6.7	6.8	6.2
	7	9.7	19.2	15.9	12.6	10.1	9.4	10.1	9.5	9.7	9.0	10.0
	21	7.5	6.0	8.3	17.2	15.1	6.7	6.1	6.6	5.6	5.5	5.4
	24	9.9	8.7	13.8	13.8	9.3	8.0	8.8	10.2	9.4	8.9	9.4
neu	2	2.35	2.32	5.39	12.6	11.9	5.95	6.56	4.63	3.38	3.11	3.39
	4	0.90	0.68	4.12	4.18	2.38	1.50	1.23	1.86	1.30	1.59	1.72
	5	2.70	1.56	4.45	10.4	7.63	3.21	2.57	2.15	1.94	1.43	1.60
	7	5.99	5.08	12.9	8.19	6.05	3.64	3.29	3.12	3.34	3.78	2.97
	21	2.40	1.56	4.90	11.8	11.4	4.25	2.89	2.64	1.65	1.87	1.65
	24	3.81	3.61	8.00	6.83	3.42	3.58	2.80	3.21	3.77	3.21	2.63

WBC white blood cells **neu** neutrophils + time 10.00 hours # 16.00 hours

Appendix 4.5

Individual trace mineral concentration of calves infected with *P .haemolytica* A 1 isolate (SB/82/1)

		Day of the experiment									
		-3	-2	-1	0	0	1	1	2	3	4
Element	**Calf**	+	+	+	+	#	+	#	+	+	+
Iron	2	19.03	27.20	26.43	25.24	24.58	7.05	6.23	4.95	8.94	8.63
	3	28.42	26.17	27.88	30.33	25.18	6.98	6.04	*		
	8	26.21	24.95	28.50	23.03	23.79	7.54	5.66	4.41	7.75	5.34
	9	30.99	26.24	26.73	26.94	26.36	7.08	**			
	14	22.56	22.70	30.56	41.20	33.19	5.13	5.33	6.40	22.44	19.31
	21	25.90	23.48	30.31	30.61	20.82	6.47	10.32	18.81	14.23	22.69
Zinc	2	11.09	17.00	16.35	15.51	3.90	5.42	6.80	5.89	9.86	11.84
	3	12.95	11.95	11.81	11.81	10.38	5.46	9.70	*		
	8	15.06	13.06	15.37	13.04	4.94	3.90	7.33	4.05	5.53	7.89
	9	14.80	14.31	13.63	14.89	3.19	4.52	**			
	14	12.39	10.44	10.74	12.11	3.86	8.51	10.20	11.78	10.53	9.87
	21	11.67	13.17	13.09	15.96	4.34	7.75	9.19	9.79	9.36	10.41
Copper	2	11.72	10.92	11.01	11.31	11.50	12.33	12.43	12.15	12.39	25.86
	3	11.52	13.29	11.83	13.05	12.34	12.56	11.95	*		
	8	13.00	13.20	11.27	13.16	12.37	13.18	12.38	13.18	13.89	17.74
	9	11.57	11.34	12.21	12.56	12.31	12.83	**			
	14	14.20	13.97	11.28	12.07	12.41	13.04	13.49	11.84	11.90	12.57
	21	11.23	10.56	11.43	11.13	11.85	12.45	10.93	11.95	12.06	18.67

* died ** slaughtered on humane grounds + time 10.00 hours # 16.00 hours

Appendix 4.6

Individual cortisol concentrations (nmol l^{-1}) of calves infected with two isolates of *P. haemolytica* A1

		Day of the experiment												
		-1	0	0	1	1	2	2	3	3	4	4	5	5
Group	**Calf**	+	#	+	#	+	#	+	#	+	#	+	#	+
I	2	21.3	55.6	79.0	59.1	79.8	82.6	145	122	176	143	138	163	
Isolate	3	11.6	19.1	30.3	48.7	75.1	*							
(SB/82/1)	8	13.8	3.42	15.5	88.2	71.3	92.9	67.5	33.1	44.7	79.3	58.4	87.2	159
	9	25.7	17.4	22.4	28.0	**								
	14	26.3	21.7	23.9	51.2	32.7	55.7	80.5	61.3	94.3	74.8	84.2	109	100
	21	21.8	15.0	23.9	27.5	22.4	37.4	30.2	36.1	40.3	39.5	48.6	48.1	54.4
II	2	19.5	18.2	17.8	34.6	41.0	27.1	39.0	33.2	33.9	28.7	24.5	21.9	29.7
Isolate	4	17.8	14.3	18.4	42.3	39.0	33.2	36.9	23.4	41.3	35.4	43.3	36.4	38.56
(SC/82/1)	5	16.3	14.8	17.4	48.3	51.2	36.5	23.5	31.8	39.3	33.1	32.0	28.7	41.34
	7	15.3	15.2	20.3	49.2	44.8	51.3	41.2	44.3	45.3	41.0	51.6	45.6	44.67
	21	17.9	18.8	21.2	18.2	18.7	17.8	22.1	19.5	22.3	19.8	17.3	19.8	16.4
	24	14.9	17.3	19.8	18.5	22.0	19.8	20.4	18.5	17.4	18.2	20.6	17.8	18.3

* died ** slaughtered on humane grounds + time 10.00 hours # 16.00 hours

Appendix 4.7

Individual thyroxine concentrations (nmol l^{-1}) in calves infected with *P. haemolytica* A 1

		Day of the experiment								
		-1	0	0	1	1	2	3	4	5
Group	**Calf**	+	+	#	+	#	+	+	+	+
I	2	61.2	74.0	57.7	14.1	13.9	18.6	24.5	22 .1	25.2
Isolate	3	56.3	61.7	69.3	17.5	5.0	*			
(SB/82/1)	8	89.3	75.1	79.5	24.8	27.1	17.5	32.7	23.1	25.8
	9	79.5	74.6	44.2	12.3	**				
	14	59.0	63.1	61.0	20.3	31.9	29.8	34.3	25.1	52.1
	21	62.3	58.7	57.2	31.3	17.0	26.7	41.2	38.3	29.9
II	2	55.6	57.3	43.2	28.6	26.8	42.5	34.5	46.7	44.4
Isolate	4	51.2	56.3	48.6	30.3	35.8	29.0	46.8	62.5	66.8
(SC/82/1)	5	53.6	55.1	52.1	29.5	42.5	46.2	60.3	55.4	56.4
	7	62.4	61.2	44.3	25.8	32.4	38.8	48.2	60.4	68.3
	21	58.2	55.7	62.1	35.3	39.4	66.5	66.3	64.8	71.5
	24	64.7	63.2	78.3	47.1	55.2	49.3	64.3	77.5	72.5

* died ** slaughtered on humane grounds + time 10.00 hours # 16.00 hours

Appendix 4.8

Individual haptoglobin concentration (g l^{-1}) of the calves infected with *P. haemolytica* A1

		Day of the experiment												
		-1	0	0	1	1	2	2	3	3	4	4	5	5
Group	Calf	+	+	#	+	#	+	#	+	#	+	#	+	#
I	2	0.20	0.22	0.31	1.51	2.08	2.51	1.25	1.80	0.98	0.83	0.72	0.54	0.52
Isolate	3	0.18	0.18	0.21	1.60	2.38	*							
(SB/82/1)	8	0.18	0.21	0.25	1.67	2.15	2.37	1.71	1.70	0.93	0.91	0.87	0.50	0.54
	9	0.18	0.20	0.28	2.08	**								
	14	0.27	0.19	0.23	1.02	1.45	1.64	1.41	1.07	0.89	0.63	0.57	0.53	0.54
	21	0.22	0.25	0.28	1.34	1.35	1.67	1.20	0.94	0.86	0.72	0.55	0.51	0.52
II	2	0.18	0.20	0.19	2.03	2.92	2.11	2.26	2.51	2.79	2.04	1.18	1.09	0.65
Isolate	4	0.28	0.27	0.32	1.92	2.56	2.45	2.06	2.12	1.51	1.75	2.02	1.59	0.56
(SC/82/1)	5	0.18	0.23	0.24	1.66	2.87	2.80	2.56	1.13	1.05	0.76	0.65	0.55	0.69
	7	0.52	0.56	0.62	1.92	2.22	2.35	1.34	2.12	1.61	1.68	1.72	1.07	0.62
	21	0.18	0.22	0.23	1.71	1.99	1.82	0.30	2.78	2.05	1.51	1.24	0.66	0.67
	24	0.18	0.20	0.24	0.27	0.37	0.37	0.26	0.13	0.08	0.18	0.11	0.16	0.13

* died ** slaughtered on humane grounds + time 10.00 hours # 16.00 hours

Appendix 4.9

Individual Serum amyloid A concentration (ug ml-[1]) in calves infected with *P. haemolytica* A1

		Dayof the experiment												
		-1	0	0	1	1	2	2	3	3	4	4	5	5
Group	**Calf**	+	+	#	+	#	+	#	+	#	+	#	+	#
I	2	3.4	3.3	10.3	97.5	105	140	124	111	59	98	61	68	39
Isolate	3	3.2	3.4	7.8	88.8	115	*							
(SB/82/1)	8	3.4	3.4	8.9	84.0	114	150	142	97	72	82	60	72	42
	9	3.3	3.1	11.3	61.3	**								
	14	2.9	3.2	6.4	54.1	98	119	107	102	42	74	70	57	31
	21	3.3	3.1	5.2	77.2	101	123	111	83	55	64	66	40	28
II	2	4.0	3.3	7.2	10.2	55.3	85.7	45.2	40.3	30.1	9.4	10	7.4	8.8
Isolate	4	3.1	3.0	4.0	30.5	95.4	90.4	80.2	40.0	35.4	9.0	6.5	7.4	5.8
(SC/82/1)	5	3.2	3.1	5.3	65.3	50.9	45.5	40.5	15.6	11.7	9.2	9.2	8.2	7.6
	7	7.5	5.7	11.2	97.1	95.2	90.4	88.2	67.0	43.2	11.3	7.7	6.5	5.5
	21	5.6	5.0	9.0	45.6	75.2	110	70.2	64.0	36.7	9.8	6.7	5.9	7.8
	24	3.1	3.4	7.6	12.4	23.5	24.5	10.3	9.0	5.4	4.0	3.0	3.2	3.5

* died ** slaughtered on humane grounds + time 10.00 hours # 16.00 hours

Appendix 4.10.

Individual fibrinogen concentration of the calves infected with *P. haemolytica* A 1

		Day of the experiment										
		-1	0	0	1	1	2	2	3	3	4	4
Group	**Calf**	+	+	#	+	#	+	#	+	#	+	#
	2	409	370	165	909	769	1176	1666	909	666	1176	1176
II	4	514	345	454	909	909	909	769	666	500	909	561
Isolate	5	367	408	370	666	909	909	1176	909	909	1176	909
(SC/82/1)	7	600	666	769	1666	1176	1176	1176	666	1176	909	1666
	21	367	263	370	769	2500	1666	909	909	1666	1666	1769
	24	692	408	561	666	666	345	666	1666	666	909	561

Appendix 4.11.

Individual ceruloplasmin concentration of the calves infected with *P. haemolytica* A 1

		Day of the experiment									
		0	0	1	1	2	2	3	3	4	5
Group	**Calf**	+	#	+	#	+	#	+	#	+	+
	2	0.09	0.09	0.09	0.09	0.08	0.09	0.17	0.18	0.12	0.16
I	3	0.08	0.08	0.10	0.11	*					
Isolate	8	0.12	0.14	0.14	0.12	0.15	0.12	0.23	0.23	0.25	0.31
(SB/82/1)	9	0.11	0.11	0.10	**						
	14	0.11	0.11	0.13	0.15	0.14	0.11	0.10	0.15	0.13	0.14
	21	0.11	0.12	0.12	0.12	0.16	0.15	0.12	0.16	0.15	0.23
	2	0.14	0.13	0.14	0.13	0.14	0.11	0.13	0.11	0.13	0.14
II	4	0.14	0.14	0.14	0.15	0.14	0.14	0.13	0.14	0.13	0.14
Isolate	5	0.14	0.14	0.14	0.13	0.14	0.11	0.13	0.11	0.14	0.14
(SC/82/1)	7	0.13	0.14	0.14	0.12	0.14	0.09	0.14	0.17	0.14	0.15
	21	0.14	0.14	0.14	0.16	0.14	0.10	0.14	0.15	0.13	0.13
	24	0.14	0.14	0.14	0.11	0.14	0.16	0.13	0.12	0.13	0.13

* died ** slaughtered on humane grounds

Appendix 4.12

Pulmonary consolidation score (%) of calves infected with *P. haemolytica* A 1

Group	Calf	Dorsal	Ventral	Total	Day
	2	78.8	77.1	78.0	6
I	3	57.3	57.9	57.6	2
Isolate	8	85.2	90.4	87.8	6
(SB/82/1)	9	46.7	48.9	47.8	1
	14	35.2	45.4	40.3	6
	21	35.1	46.4	40.9	6
	2	0.00	2.29	1.15	6
II	4	6.75	14.48	10.62	6
Isolate	5	6.53	5.45	5.99	6
(SC/82/1)	7	12.27	15.84	14.06	6
	21	6.19	14.76	10.48	6
	24	22.78	16.30	19.54	6

Appendix 5.1

RPM I Tissue Culture Medium

Sterile distilled water	431.5 ml
RPM1 1640	500.0 ml
glutamine (200mM)	5.0 ml
penicillin (5000 U ml $^{-1}$)streptomycin/5000 μg ml^{-1}	5.0 ml
Sodium bicarbonate (7.5%)	13.5 ml

GLOSSARY

AGP	α-acid glycoprotein
AFC	affinity chromatography
APbS	acute phase bovine serum
apo AI	apolipoprotein-AI
apo AII	apolipoprotein-AII
APP	acute phase protein(s)
APR	acute phase response
apo	apolipoprotein(s)
BAL	bronchoalveolar lavage
BCA	bicinchoninic acid
BPP	bovine pneumonic pasteurellosis
BSA	bovine serum albumin
b-AA	bovine amyloid A
b-SAA	bovine-serum amyloid A
°C	centigrade (celsius)
Ci	curie
cm	centimetre
Cp	ceruloplasmin
cpm	counts per minute
CRP	C-reactive protein
Cu	copper
Da	Dalton
DEAE	diethylaminoethyl
dH_2O	distilled water
dl	decilitre
EDTA	ethylenediaminetetra-acetate
ELISA	enzyme link immunosorbent assay
FBS	foetal bovine serum
Fe	iron
FPLC	fast protein liquid chromatography
g	gram
GF	gel filtration
Hb	haemoglobin
Hct	haematocrit
h	hour
HRP	horseradish peroxidase
HDL	high density lipoprotein(s)
HIC	hydrophobic interaction chromatography
Hp	haptoglobin
IHA	Indirect haemagglutination test
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
k	kilo
kD	kilo Dolton
l	litre
LDL	low density lipoprotein(s)

mg	milligram
min	minute(s)
ml	millilitre
mm	millimetre
mM	millimole
mmol	millimole
mRNA	messenger ribonucleic acid
M_r	molecular radius
N	normality
ng	nanogram
nm	nanometre
nmol	nanomole
OD	optical density
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween 20
PPD	*p*-phenylenediamine
RIA	radioimmunoassay
rpm	revolutions per minute
SAA	serum amyloid A
SD	standard deviation
SEM	standard error of mean
SDS	sodium dodecyle sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
T_4	thyroxine
TBS	tris-buffered saline
TCM	tissue culture medium
TSB	trypticase soy broth
TEMED	N',N',N',N',-tetramethylenediamine
TNFα	tumour necrosis factor- alpha
TNF_β	tumour necrosis factor- beta
UF	ultra centrifugation
µ	micro
µg	microgram
µl	microlitre
µmol	micromole
v/v	by volume
w/v	by weight
Zn	zinc

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