THE EFFECTS OF A LOCALIZED *PASTEURELLA HAEMOLYTICA* INFECTION ON THE ERYTHROMYCIN BINDING PROPERTIES AND CONCENTRATION OF BOVINE ALPHA-1-ACID GLYCOPROTEIN IN SERUM AND TISSUE CHAMBER FLUID

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CHAPTER I

INTRODUCTION

Alpha-1-acid glycoprotein (AAG) is an acute phase protein which plays a role in non-specific immunosuppression. Serum concentrations of AAG increase during episodes of infection or inflammation, and could affect the clinical efficacy of drugs by binding to them and inhibiting their tissue distribution or *in vivo* activity. Drugs which are organic bases are chiefly bound by AAG and are, therefore, most susceptible to the effects of higher serum concentrations of AAG.

Most infections are not limited to the blood vascular system but are located in tissues. Considering that antimicrobial agents are inactivated when they are bound to proteins, the concentrations of drug binding proteins in interstitial fluids of tissues and the degree to which drugs are bound in interstitial fluids are of considerable relevance to *in vivo* antibacterial efficacy. Unfortunately, the interstitial fluid space is relatively inaccessible and direct measurement of protein concentrations and drug binding is difficult.

Tissue chambers are hollow, perforated devices that have been utilized as models to study interstitial fluid and

tissue penetration of xenobiotics. After implantation in tissue, the chambers form a cavity which is in direct communication with the interstitial space and fills with a transudate that is thought to be similar to interstitial fluid. Implanted tissue chambers, therefore, provide a model which can be used to investigate the interactions between AAG and drugs in the interstitial space.

In this study, concentrations of AAG were measured in bovine serum and in tissue chamber fluid before and at intervals after inoculation of the tissue chambers with Pasteurella haemolytica. In addition, parameters describing binding of erythromycin in bovine serum and tissue chamber fluid and to the pure proteins, AAG and bovine serum albumin, were determined.

CHAPTER II

LITERATURE REVIEW

Alpha-1-Acid Glycoprotein

The liver plays an important role in the systemic reaction to tissue injury and inflammation. This reaction, termed an acute phase response, is associated with clinical conditions such as cancer, arthritis, infection, trauma, and surgery. The acute phase response is characterized by an increased production and secretion of plasma proteins (>25% above normal plasma concentrations), termed acute phase proteins (Rosen, 1990; Kushner, 1982). These proteins include fibrinogen, C-reactive protein, serum amyloid A-protein, haptoglobin, and a carbohydrate residue-containing protein called alpha-1-acid glycoprotein (AAG).

Physicochemical Properties of AAG

Glycoproteins consist of carbohydrate side chains linked to a central protein core (Winzler, 1960). First isolated from serum in 1882 (Landwehr, 1882), AAG was initially termed seromucoid, owing to the similarities between it and a mucoid protein found in eggs (Freund, 1892). More recent research, conducted on the human molecules, has focused upon characterization (mainly determination of

molecular weight) and isolation (Freund, 1892; Landwehr, 1882; Weimer et al, 1950; Schmid, 1955; Bezkorovainy & Winzler, 1961) and has resulted in the current designation of alpha-1-acid glycoprotein.

Studies investigating the molecular characteristics of AAG have reported molecular weights ranging from 34,100 (Kawahara et al, 1973) to 54,000 (Easton et al, 1962). Wide differences in observed physical and chemical properties of AAG have been attributed to variation in methods of isolation (Alper, 1974; Busby & Ingham, 1986; Hao & Wickerhauser, 1973) and the degree to which the chosen method affected glycosylation of the core protein. Using a two step method with adsorption on a diethylaminoethyl (DEAE) Sephadex® column and ion-exchange chromatography of the DEAE eluate on a carboxymethyl (CM) cellulose column to isolate the native molecule, the molecular weight of bovine AAG was determined to be 44,100 (Sigma Chemical, St. Louis).

Several studies have been conducted comparing AAG molecules from different species. A study comparing bovine AAG with that of humans (Bezkorovainy & Doherty, 1962) revealed only minor differences between the two proteins. Findings by Anantha Samy et al (1965) comparing the glycoproteins isolated from sheep, goat, cow, buffalo and monkey. The proteins exhibited similar properties on electrophoresis, ultracentrifugation, and in molecular weight. There were differences in chemical composition, mainly in the carbohydrate portions. A study has also been reported

comparing AAG composition in the same species, but located in different parts of the body. Bezkorovainy (1965) compared AAG from bovine serum, colostrum, and milk whey. The AAG molecules from serum and colostrum were very similar, but milk contained very small amounts of a carbohydrate deficient AAG molecule.

Effect of Disease on Alpha-1-Acid Glycoprotein Concentrations in Humans

Numerous studies have reported that patients with clinical conditions associated with inflammation have increased serum concentrations of AAG. Harshman et al (1974) performed serial AAG assays on samples collected from 213 patients with cancer. Patients with active metastasis or clinical disease exhibited AAG concentrations of up to 240 mg/dl. Patients in clinical remission had lower AAG serum concentrations of between 64 to 86 mg/dl. Mean normal AAG concentrations in male and female humans are reported to be 59.9 mg/dl and 52.4 mg/dl, respectively (Yost & DeVane, 1985). Arthritis sufferers had serum AAG concentrations of 149 ± 60 mg/dl, and patients with Crohn's disease (regional ileitis) had a mean of 165, as compared with the control mean of 66 mg/dl (Piafsky et al, 1978). A study investigating the concentrations of AAG in burn patients revealed a mean level of 222 mg/dl, in comparison with a normal of 83 mg/dl (Bloedow et al, 1982). Trauma patients had increases from 70 mg/dl to peak values of 243 mg/dl at 10 to 14 days

after the insult, and patients suffering from myocardial infarction showed increases from 69 ± 9 mg/dl to 181 ± 69 mg/dl post infarction (Freilich & Gardina, 1984).

The prognostic value of AAG concentrations is illustrated by the observation that neonates with infections that have a poor prognosis have higher serum concentrations (167 ± 67 mg/dl) than those with a favorable prognosis (133 ± 75 mg/dl). Concentrations of AAG considered normal for human neonates vary between 18 ± 8 mg/dl at birth and 52 ± 8 at 2 days of age (Sann et al, 1984).

Certain diseases may cause decreased AAG serum concentration: liver cirrhosis sufferers have serum AAG concentrations that are only 30% of healthy controls, probably due to a decreased capacity of the diseased liver to synthesize and secrete AAG (Barre et al, 1984).

Increased AAG production following inflammation is mediated via leukocyte endogenous mediator and/or interleukin 1. These cytokines are released by inflammatory cells and pass to the liver where they induce transcription and synthesis of AAG (Schreiber, 1987).

Inflammation also leads to endocrine changes which contribute to the acute phase response. In particular, thyroid stimulating hormone and thyroxin rates of synthesis are increased (Kushner, 1982), resulting in increased levels of thyroid hormones, which have a stimulatory effect on AAG synthesis. In addition, although steroids inhibit the production of interleukin-1 (Ganong, 1989), they have a

direct stimulatory effect on the production of AAG (Kulkarni et al, 1985).

Alpha-1-acid glycoprotein is also produced by bovine lymphocytes, numbers of which are increased during infection and inflammation. However, the relatively small amounts (nanograms per million lymphocytes) produced by lymphocytes probably contribute little to increased plasma concentrations resulting from inflammation (Iwata et al, 1988a). Alpha-1-acid glycoprotein molecules circulate in the blood until the terminal sialic acid is lost, and they are then taken up by hepatocytes during a single pass through the liver (Charlwood et al, 1979).

Quantitation of AAG

Several methods have been employed to measure the concentration of AAG. Enzyme linked immunosorbant assay (ELISA) (Vittinghus, 1990; Freyman et al, 1987) and radial immunodiffusion (RID) (Tamura et al, 1989; Iwata et al, 1988b; Yost & DeVane, 1985; Iwata et al, 1989) have been used in several studies to measure AAG serum and urine concentrations. Less common methods include rocket electrophoresis (Dello et al, 1988), nephelometry (Colley et al, 1983), and precipitation of the protein and detection using bicinchoninic (BCA) protein reagent (Conner et al, 1988; Conner et al, 1989). The RID method relies on the principle that molecules smaller than 200,000 M.W. are able to diffuse readily through the agarose gel. As the antigen

diffuses radially from the well punched in the gel, which contains a uniform concentration of a specific antibody, the concentration of antigen decreases until an optimal ratio of antigen to antibody concentrations is reached, resulting in the formation of an antigen-antibody complex and precipitation of that complex. Thus, a precipitin ring is formed around the well (Clausen, 1969). Studies revealing a linear relationship between the diameter of the precipitin ring and the concentration of antigen, without data transformation, have been conducted by Mancini et al (1965).

Function of AAG

Although many functions of AAG are yet to be elucidated, some of the activities of AAG that have been identified include; inhibition of malaria and erythrocyte interaction, transformation of lymphocytes, and inhibition of phagocytic activity of macrophages. The carbohydrate portion of the molecule apparently interacts with lymphoid cell surfaces, leading to non-specific immune suppression (Schrieber, 1987).

Binding properties of AAG and the Effects of Binding on Drug Distribution and Efficacy

AAG binds to a wide range of ligands including endogenous steroids (Ganguly et al, 1967) and xenobiotics (Kremer et al, 1988). However, it appears to have a particular

affinity for compounds which are organic bases. Unbound lidocaine concentration was found to be inversely related to the concentration of serum AAG (Routledge et al, 1980; Edwards et al, 1982). Free serum concentrations of many Beta-adrenocepter drugs decrease in patients with arthritis (Belpaire et al, 1982), and methadone free drug concentrations were appreciably lower in patients with cancer than in controls (Romach et al, 1982; Abramson, 1982). Meperidine binding was studied in maternal and fetal plasma and was found to be higher in maternal plasma, which contained higher concentrations of AAG (Nation, 1981). Concentrations of unbound propranolol were higher in younger patients than in older patients. Again, concentrations of AAG were higher in the older subjects (Tenero et al, 1990). Piafsky et al (1978) has reported similar results in a previous study, as has Abramson et al (1982). A comprehensive review of the many studies addressing these correlations has been published by Kremer et al (1988).

Alpha-1-acid glycoprotein also has affinity for some acidic drugs; drugs containing no trace of a carboxyl moiety are more likely to bind to AAG than other acidic xenobiotics (Urien et al, 1982).

Several studies have focused on identifying and measuring numbers of binding sites on AAG and the affinity with which AAG binds xenobiotics. Research by Westphal & Ashley (1962) indicated that estradiol interacted at 3 binding sites. In a later study, Kerkey & Westphal (1968) reported

that estradiol interacted at 7 binding sites on AAG and that cortexone, cortisol, progesterone, and testosterone associated with a single site. Dette et al (1982) found a single primary site of binding for erythromycin binding in humans, and in a later study revealed that the primary binding protein for erythromycin in human serum was AAG (Dette & Knothe, 1986). A recent report characterizing the drug binding sites on AAG using florescent probes has demonstrated that the binding of basic drugs to AAG inhibits the binding of both basic and acidic probes. Acidic drugs (those having a binding affinity for AAG) displaced acidic probes from the AAG molecule but did not affect the binding of basic probes. These data suggest that there is one binding site that interacts with both acidic and basic drugs. Based upon these results, Maruyama et al (1990) concluded that AAG has a wide binding site, to which both acidic and basic molecules bind, and that the regions that bind acidic and basic drugs overlap to a significant degree.

Drug binding becomes a significant factor when considered in the light of pharmacokinetics. A drug that is bound to a serum protein is restricted to the blood vascular system. Thus, binding to plasma proteins may affect the total body clearance of a drug. Furthermore, there is an increasing body of evidence indicating that protein-bound xenobiotics are not pharmacologically active (Shoeman et al, 1973).

Several techniques have been employed to investigate

protein and drug interactions. Techniques available can be divided into spectroscopic and non-spectroscopic methods. Spectroscopic methods include visible, ultraviolet, and florescence spectroscopy along with nuclear magnetic resonance, electron spin resonance, optical rotary dispersion and circular dichroism. These methods determine the characteristics of the ligand-macromolecule complex. Non-spectroscopic techniques include high performance liquid chromatography (HPLC), ultracentrifugation, ultrafiltration, and equilibrium dialysis. These techniques are based upon the determination of the free (unbound) drug concentration (Sebille, 1990). The most commonly used methods are HPLC, ultrafiltration, and equilibrium dialysis.

Equilibrium dialysis is, in theory, the most accurate way to determine the percentage of binding because the equilibrium is not disturbed by shifts in volumes of protein-free and protein-containing compartments. The semipermeable membrane which separates protein-free and protein-containing compartments allows diffusion of drug molecules with molecular weights below a maximum limit. Equilibrium or "steady state" is reached when rates of drug diffusion in both directions across the membrane are equal. At this stage the amount of free drug is equal in both compartments. However, drug bound to protein is sequestered in the protein containing compartment. The amount of drug bound to protein is determined by measuring the total amount of drug in the protein compartment (free plus bound drug) and in the drug

compartment (free drug). Since the amount of free drug in both compartments is equal, the difference between the concentrations of drug present in the two compartments is equal to the concentration of drug bound to protein. Parameters describing the affinity constant and the number of binding sites per molecule can be estimated by a Scatchard plot (Scatchard, 1948). A model for analyzing protein-drug interaction systems in which the geometric coordinates are independent of the concentration of the binding macromolecules has been presented by Rosenthal (1967). If protein binding is saturated, the affinity constant and the protein concentration may be estimated by solving the quadratic equation describing the relationship between total and free drug concentrations (Borne, 1989).

Effect of Disease on AAG concentrations in Non-human Species

A study of bovine AAG by Tamura et al (1989) resulted in an estimation of molecular weight of 42,000 ± 2,000 and a carbohydrate content of 26.6%.

Iwata et al (1988b) reported that the normal serum level of AAG in the adult bovine was 31 ± 9 mg/dl. However, concentrations in suckling and yearling animals were slightly lower (25 ± 11 mg/dl and 27 ± 9 mg/dl, respectively) and concentrations in fetuses (where the liver is not yet fully developed) were even lower (11 ± 4 mg/dl).

Alpha-1-acid glycoprotein levels in cattle with

pneumonia averaged 64 ± 45 mg/ml (roughly double the average serum concentration), and in those with malignant lymphoma had AAG concentrations were as high as 355 mg/dl, 8 times the normal serum concentration (Iwata et al, 1988b). Research by Iwata et al (1989) also revealed that serum AAG concentrations of bovines suffering from leukemia were approximately double normal levels. Another study recorded AAG serum concentrations in calves that were infected with Pasteurella haemolytica, Ostertagia ostertagi, or administered endotoxin. After endotoxin administration, the concentration of AAG increased to 150% of the normal levels, reaching a peak on day 4. Pasteurella haemolytica infection resulted in a doubling of the normal concentrations, with peaks occurring on day 4. Ostertagia infections did not result in any changes in acute phase proteins (Conner et al, 1989).

Subcutaneous injection of turpentine causes an acute phase response which is similar to that following infectious agents. Serum concentration of AAG started increasing on day 2 post injection, peaked on day four at roughly 3 times the normal level, and then started decreasing, reaching normal concentrations by day 17. Concentrations for other acute phase proteins, alpha-1-antitrypsin, ceruloplasmin, fibrinogen, and haptoglobin were similar to those observed in human acute phase responses, with the exception of C-reactive protein, which is not an acute phase protein in the bovine. (Conner et al, 1988). Animals suffering from

traumatic pericarditis, arthritis, mastitis, pneumonia, or mesenteric liponecrosis experienced increases in serum AAG concentration of 100%, 100%, 91%, 70%, and 43%, respectively (Tamura et al, 1989).

Similar observations have been made in species other than the bovine. Shibata et al (1977) reported that in rats inflammation lead to a 5 to 6 fold increase in serum AAG. In dogs suffering from an inflammatory disease, there was again a 5 to 6 fold increase in serum AAG levels (Dello et al, 1988).

Tissue Chambers

Techniques for Measuring Drug Concentration in Tissues

Except in the case of bacterial infections of the blood vascular system, or the cells of the hematopoietic or lymphatic system, the majority of infections are located in the interstitial spaces of tissues. Thus, it is important to have accurate estimates of the concentrations of unbound antibiotic drugs in the interstitial space. Methods for estimation of the tissue penetration of drugs have been reviewed (Clarke, 1989b) and include; skin blisters, skin windows, subcutaneous disc model, fibrin clots, fluids from body spaces, and exudates from surgical incisions. Skin blisters may be induced by suction or by irritants such as cantharidin, skin windows are produced by abrading the epidermis to expose the dermis, and then allowing

equilibrium to be established between paper discs or saline filled chambers applied to the abraded dermis. Skin windows are similar to the subcutaneous disc model, except that in the case of the latter model tissue fluid is sampled from subcutaneous tissue instead of the dermal surface. Models utilizing fibrin clots involve subcutaneous implantation of clots in rabbits to study drug penetration into the tissues. These clots have lower peak concentrations and longer elimination half lives than the corresponding values measured in serum. Fluids from body spaces have also been assayed, but long diffusional differences and the nature of membranes lining the cavities make results from these studies applicable only in cases of infection of the cavities (peritonitis, pleuritis, pericarditis, or tenosynovitis). Similarly, results from surgical incision exudates are applicable only to suture line infections, because bleeding and inflammation alter the drug distribution from what it would be in normal interstitial spaces. Assay of peripheral lymph has been used to give estimates of penetration of drugs into the interstitial space. However, problems with penetration of protein bound molecules through the lymphatic endothelium, changes in lymph flow, and difficulties in cannulation of lymphatics can result in unreliable estimates. In addition, thoracic duct lymph is unusable because of a high protein content (in contrast to the low protein content of interstitial fluid) and the large amounts of material gathered from the organs that metabolize and excrete drugs (liver,

kidneys, gastrointestinal tract). Tissue homogenates have been used to estimate drug penetration. However, homogenates contain blood and intracellular fluid in addition to interstitial fluid. Estimates of drug penetration must be adjusted to compensate for the presence of the other components. However, the technique is appropriate for measurement of drug residues in food animals. Volume of distribution is still very commonly used for the estimation of penetration of drug outside the blood vascular system. The assumption is made that the drug is distributed evenly throughout its volume of distribution, but this is seldom the case. For example, aminoglycosides are noted for their accumulation in kidneys (Katzung, 1989). Implanted tissue chambers have also been used to measure drug penetration into the extravascular region, and have the advantage of being easy to sample. Multiple chambers can be implanted for studies that require frequent sample collection (Ziv et al, 1982).

Construction and Utilization of Tissue Chambers

Tissue chambers are hollow, perforated devices that have been constructed of various materials including stainless steel, plastic (golf 'whiffle' balls), and Delrin®. Tissue chambers have been implanted subcutaneously (Short et al, 1987), into the peritoneal cavity (Gerding et al, 1976) and in organs such as the prostate (Eikenburg et al, 1976b)

and the kidney (Eikenburg et al, 1976a). Initially used to measure pressures in the interstitial space by Guyton (1963), later studies have used the tissue chamber to attempt to estimate drug penetration into the interstitial space (Bengsston et al, 1986), to measure the characteristics of microbial growth in vivo (Clarke et al, 1989d), and to study the pathophysiology of inflammation (Clarke et al, 1989a).

The tissue chamber model used in the current study was developed and validated by Clarke and coworkers (1989a; 1989b; 1989c; 1989d). This perforated thermoplastic tissue chamber was constructed of Delrin®, and the open end of the shallow cylinder was covered with a silicone rubber membrane (Silastic®). After subcutaneous implantation of the chambers in cattle, the chambers fill with blood, inflammatory cells, loose fibrin strands, and protein. With the passage of time, fibroblasts invade the tissue chamber and begin deposition of a loose granulation tissue that becomes more organized until it forms a lining of the tissue chamber. The fluid inside the tissue chamber immediately after implantation is serosanguinous. With time, the number of erythrocytes in the chamber fluid decreases, the concentration of sodium and potassium increases, and the amount of protein decreases. Thus, by day 60, the fluid composition in the tissue chambers approximates the hypothetical composition of interstitial fluid (Clarke et al 1989a), making the tissue chamber a viable model for pharmacokinetic and

other studies.

Studies using subcutaneously implanted tissue chambers to measure the concentrations of oxytetracycline in cattle (Bengtsson et al, 1986) and cephapirin in horses (Short et al, 1987) concluded that tissue chambers are more suited as a model for measuring drug concentrations in regions of limited access (abscesses, joint capsules) than as a model for the extravascular space of highly perfused tissues. Studies conducted by Clarke et al (1989c; 1989d; 1992) focused on the use of tissue chambers as a model for studying the effect of P. haemolytica infection on the tissue distribution and in vivo efficacy of antibacterial agents. Inoculation of P. haemolytica into tissue chambers implanted subcutaneously in cattle results in the establishment of a soft-tissue infection model, which can be used for many applications, including the study of protein-drug interactions.

CHAPTER III

ASSAY OF ALPHA-1-ACID GLYCOPROTEIN

Introduction

Alpha-1-acid glycoprotein (AAG) is an acute-phase protein produced by the liver (Schreiber, 1987). Serum concentrations of acute-phase proteins increase in response to localized inflammation or infection (Kushner, 1982), systemic disease states (Harshman et al, 1967, Kremer et al, 1989), exposure to toxins (Conner et al, 1988), and trauma (Neuhaus et al, 1966). Raised serum levels of AAG have been detected in calves following intratracheal inoculation with Pasteurella haemolytica (Conner et al, 1989).

The effect of P. haemolytica on serum concentrations of AAG has important implications with respect to therapeutic management of pneumonic pasteurellosis, because the disposition and therapeutic efficacy of antibacterial agents used to treat the infection may be altered. In contrast to albumin, which usually binds acidic drugs, AAG binds many basic drugs with high affinity (Kremer et al, 1988). Antibacterial agents bound to serum proteins may be prevented from distributing to peripheral tissues or may be rendered inactive. Since antibacterial efficacy is most closely correlated with drug concentrations in the interstitial

space, where most infecting bacteria infections are located, interstitial concentrations of AAG are of particular interest (Wise et al, 1980, Wise, 1986).

Subcutaneous tissue chambers have been used to study interstitial fluid (Guyton, 1963) and to estimate tissue concentrations of antibacterial agents (Bengtsson et al, 1986). After implantation, these chambers become vascularized by infiltrating granulation tissue. Fluid that accumulates in the chambers is believed to be similar in composition to interstitial fluid (Clarke et al, 1989a). A soft-tissue infection model can be established by inoculation of P. haemolytica into subcutaneous tissue chambers (Clarke et al, 1989c; 1989d; 1992), thus providing a convenient method of studying the effect of P. haemolytica infection on concentrations of AAG in tissue fluids.

Several different methodologies have been employed to measure concentrations of AAG, which has a molecular weight of 42,000 ± 2000 and a carbohydrate content of approximately 26% (Tamura et al, 1989). Recently, an accurate radial immunodiffusion assay (RID) was used to quantify AAG levels in bovine serum. Antiserum used in the RID was obtained by immunizing rabbits with AAG purified from bovine serum by precipitation, ion-exchange chromatography and gel filtration (Iwata et al, 1987). Current availability of commercially purified bovine AAG now makes lengthy protein extraction and purification steps unnecessary.

The objectives of this study were to develop a single

radial immunodiffusion assay using commercially prepared AAG and to use this assay to measure the concentrations of AAG in serum and tissue chamber fluid before and after inoculation of tissue chambers with P. haemolytica.

Materials and Methods

Animals

Ten Hereford cross-bred calves 4 to 8 months of age and weighing from 100 kg to 225 kg were used in this experiment. Prior to use, the animals were dewormed with fenbendazole (5mg/kg, Safeguard, Hoechst-Roussel) and observed on a daily basis during a two week acclimation period to establish that they were in good health. The animals were housed in small pens and maintained on a 14% protein commercial feed and alfalfa hay.

Implantation of Tissue Chambers

Two cup-shaped Delrin® (E.I. DuPont Nemours) tissue chambers, measuring 46 mm ID and 15 mm in depth, were implanted subcutaneously in each calf, one chamber in each paralumbar fossa. Perforations in the walls and base of the chambers allowed unrestricted exchange of solutes and cells between tissue chamber fluid and surrounding interstitial fluids. Chamber fluid could be aspirated by inserting a needle through the skin and a silicone rubber (Sialastic®, Dow Corning) membrane which covered the top of the chamber. Tissue chamber assembly, sterilization, surgical implantation, and collection of chamber fluid samples have been

described previously (Clarke et al, 1989b). The sterility of the surgical technique was monitored by culturing an aspirate of chamber fluid aerobically and anaerobically on 5% sheep blood agar.

Study Design

Approximately 2 months after implantation, pre-inoculation blood and chamber fluid samples were collected and all chambers were then inoculated with 1 ml of a 6-hour culture of a field isolate of P. haemolytica serotype 1 (Corstvet et al, 1973). Inocula were prepared by culture on supplemented brain heart infusion agar followed by suspension of the bacteria in phosphate buffered saline (PBS) at a concentration of 3×10^6 colony forming units/ml, as previously described (Clarke et al, 1989b). At 2, 4, 6, and 10 days after inoculation, samples of blood and tissue chamber fluid were collected. Serum and tissue chamber fluids were frozen at -20°C until the protein assay was performed.

Protein Assay

Concentrations of albumin in serum and tissue chamber fluid were determined using a bromcresol green method (Kaplan & Pesce, 1989) (Centrifichem, Baker Instruments) and analyzer (Encore, Baker Instruments).

AAG was assayed by RID. Antiserum to AAG was produced in 3 mature female New Zealand White rabbits (Clausen, 1969). Bovine AAG (Sigma Chemical) was dissolved (100 ug/ml) in 0.9% saline, the solution was emulsified with an

equal volume of adjuvant, and 1 ml of emulsion was injected intradermally in multiple sites dorsal to the scapulae of each rabbit. Freund's complete adjuvant and Freund's incomplete adjuvant were used for the first and for subsequent immunizations, respectively. Rabbits were immunized 3 times at 2 week intervals, and subsequently every 6 weeks. Every 6 weeks after the initial immunization up to 50 ml of blood was drawn from the central ear artery. Serum was harvested, clarified by centrifugation at 25,000 x g, and then filtered through a 0.5 um filter. Antiserum was frozen at -20°C for later use. Rabbits were given boosters at 6 week intervals. Blood was collected and processed every 2 weeks after subsequent immunizations.

Development of a RID assay followed standard protocols (Mancini et al, 1965). Briefly, a 1% solution of agarose (Seakem HGT, FMC Bioproducts) was prepared in PBS by heating at 80°C with stirring. The agarose was cooled to 50°C, and the antiserum was added to a final concentration of 10%. Gels were formed by pouring 37.5 ml of agarose/antiserum solution on a 125 x 200 mm plate (Gelbond, FMC Bioproducts) to a thickness of 1.5 mm. Sample wells (2.5 mm diameter) were punched in the gel and 5 ul samples of serum or chamber fluid were pipetted into each well. A series of standards (0.01, 0.03, 0.1, 0.3, 1, 3 mg/ml) in PBS were included on each gel. Gels were incubated in a humid box at 23°C for 72 hours. After incubation, gels were washed in 3 changes of PBS for 72 hours to remove unprecipitated protein followed

by 2 washes in deionized water to eliminate PBS. Gels were dried and stained (Crowle & Cline, 1977) and the diameters of the precipitin rings were measured using a vernier micrometer.

The purity of commercial bovine AAG was tested using SDS-PAGE electrophoresis (Laemmli, 1970) and a Western blot (Towbin et al, 1979) was conducted on the rabbit antiserum to confirm the specificity of the assay. Intra-assay precision was determined by measuring 5 replicates of 3 different standard concentrations on the same gel. Inter-assay precision was calculated by including identical samples in assays conducted on different gels. Three dilutions of a sample were assayed and compared with the standard curve to evaluate linearity. Sensitivity was approximated by estimating the smallest ring size which could be reliably measured. Accuracy was estimated by the addition of a known amount of AAG (0.1 mg/ml) to serum and comparing the spike concentration with the concentration calculated using standards.

Statistical Analysis

A regression analysis was conducted on successive standard curve coordinates. Accuracy and linearity were expressed as percentages. Coefficients of variability were used to describe inter- and intra-assay precision. The effects of time after inoculation on AAG and albumin concentrations in chamber fluid and serum were studied with SYSTAT, using orthogonal polynomial analysis, adjusted for

uneven spacing (Wilkinson, 1987). Protein concentration data were also analyzed by the general linear model, blocking for calves. Mean concentrations on each of the sampling days were then separated using Scheffe's test. Trends were considered significant at the $P < 0.05$ level.

Results

Log transformation of standard concentrations and ring diameters yielded a linear relationship between the highest and lowest standard concentrations (Fig. 1 pp. 55). Good linearity was demonstrated by close parallelism between the standard and sample dilutions. The smallest ring diameter which could be accurately measured corresponded to a concentration of 0.007 mg/ml. Inter- and intra-assay coefficients of variation were 9.39% and 9.48%, respectively, and the accuracy of the assay was measured at >90%.

The pre-inoculation concentrations of AAG in serum and chamber fluid (Fig. 2 pp. 56) varied widely between the ranges 0.21 - 1.10 and 0.09 - 0.66 mg/ml, respectively. Inoculation of tissue chambers caused serum concentrations of AAG to increase linearly. Concentrations of AAG in chamber fluids increased quadratically after P. haemolytica infection, with the mean peak concentration occurring at 6 days. By day 10, mean AAG concentrations in serum and chamber fluid were at least 30% greater than pre-inoculation levels (Table 1 pp. 52). Concentrations of albumin in chamber fluids increased quadratically after inoculation

with P. haemolytica (Fig. 3 pp. 57). However, serum albumin concentrations did not change. Utilizing Scheffe's test to compare means of AAG and albumin concentrations yielded a significant difference in mean concentrations of serum and chamber fluid AAG and of chamber fluid concentrations of albumin between day 0 (pre-inoculation) and day 4 (peak of the post-inoculation samples). Again, serum albumin concentrations did not change. Preliminary experiments conducted using 2 calves demonstrated that injection of sterile PBS into subcutaneous tissue chambers and subsequent sampling of serum and chamber fluid had no effect on concentrations of AAG or albumin in chamber fluid or serum pre-inoculation levels.

Discussion and Conclusions

Assay of AAG is complicated by interspecies variation in glycoprotein composition and structure, thus necessitating development of specific assays for each species. Current availability of commercially prepared bovine AAG facilitates development of such assays, by eliminating the extraction techniques previously required to isolate and purify the glycoprotein for preparation of concentration standards or production of specific antibodies. The RID assay used in the present experiment provided a convenient, sensitive, and reproducible method of measuring concentrations of bovine AAG in different biological matrices.

P. haemolytica is the primary etiological agent of

bovine respiratory disease (Jensen et al, 1976), which is responsible for considerable economic loss and animal suffering. In the absence of effective vaccines, antibacterial therapy remains a major component of control. Several of the antibacterial agents considered to be effective against P. haemolytica, such as erythromycin, are weak organic bases. Although basic drugs may associate with several plasma constituents, including albumin and lipoprotein, they are principally bound by AAG (Kremer et al, 1988). Considering that antibacterial agents are inactivated when they are bound to proteins (Wise, 1986) and that serum concentrations of AAG have been shown to increase dramatically after intratracheal inoculation of calves with P. haemolytica (Conner et al, 1989), binding of basic antibacterial agents to this acute-phase glycoprotein may reduce in vivo efficacy in diseased animals, particularly when drug concentrations achieved in the animal are only slightly higher than the minimum inhibitory concentration.

Inoculation of P. haemolytica into subcutaneous tissue chambers causes a fibrinopurulent infection with a pathogenesis which is similar to the inflammatory response following natural pulmonary infection (Clarke et al, 1989c). In contrast to interstitial fluid of pneumonic lungs, subcutaneous tissue chamber fluid is easily sampled and can, therefore, be used to estimate the concentration of interstitial constituents in P. haemolytica infected tissues. Previous experiments have shown that subcutaneous tissue chambers

form extracellular interstitial compartments (Clarke et al, 1989a) and that the composition of chamber fluid approximates the hypothetical composition of interstitial fluid (Clarke et al, 1989d). Concentrations of drug binding macromolecules in interstitial fluids are more relevant to antibacterial efficacy than blood concentrations because most bacterial infections are located in the interstitial space.

The wide intersubject variation in serum and chamber fluid AAG concentrations observed in the present study is consistent with data reported by Iwata et al (1988a), who determined that serum AAG levels in healthy yearling cattle varied within the range 0.17 - 0.50 mg/ml. AAG concentrations are affected by many conditions other than bacterial infection (Kremer et al, 1988), such as xenobiotics and viruses, and it can be assumed that apparently healthy animals may be exposed to undetected stimulants of AAG production. Furthermore, serum concentrations within individuals exhibit diurnal variations which may contribute to the wide variation in baseline values (Yost and DeVane, 1985).

The mean peak serum AAG concentration resulting from inoculation of P. haemolytica into subcutaneous tissue chambers (± 50%) was of lower magnitude than that following intratracheal inoculation (± 80%)(Conner et al, 1989), although the times taken to achieve maximum concentrations were similar (3 - 4 days). Subcutaneous injection of oil of

turpentine, which had a dose-dependant effect (Conner et al, 1988), also produced greater increases in serum AAG concentration than tissue chamber inoculation. However, an increase in AAG concentration of 50%, as was observed in the present study, may still have a profound effect on the degree of drug binding, particularly when the drug is bound with high affinity.

The concentration profile of AAG in chamber fluid was similar to that of serum, thus suggesting that AAG in interstitial fluids is derived from serum. Stimulation of the acute phase response occurs via interleukins, which induce hepatocytes and lymphocytes to synthesize proteins such as AAG (Kushner and Mackiewicz, 1987, Iwata et al, 1989b). However, lymphocytes were unlikely to be the source of AAG in infected tissue chambers because P. haemolytica inoculation causes a neutrophilic response and infected tissue chambers contain relatively few lymphocytes (Clarke et al, 1989b). Furthermore, P. haemolytica infection is known to cause erosion of blood vessels in tissue chambers, thus disrupting the integrity of the permeability barrier between blood and chamber fluid (Clarke et al, 1989a). Under such conditions, AAG could easily diffuse from blood into infected chamber fluid. The rise in chamber fluid albumin concentrations after inoculation is further evidence that plasma proteins can diffuse into infected tissue chambers.

In conclusion, P. haemolytica infection of soft-tissues stimulates production of AAG which is reflected by rises in

serum as well as tissue chamber concentrations. Higher concentrations of AAG and albumin in sites of infection may cause an increase in drug binding leading to poor efficacy and therapeutic failure.

CHAPTER IV

BINDING OF ERYTHROMYCIN

Introduction

In vivo antibacterial efficacy is determined by a number of factors including the tissue penetration of the antibacterial and activity of the antibacterial at the site of infection. Binding to serum proteins usually decreases the tissue penetration of antibacterial agents because protein-bound drugs are too large to pass through capillary endothelial layers and are therefore restricted to the circulatory compartment, away from the site of most infections. Furthermore, there is reliable evidence indicating that drugs that are protein bound are not pharmacologically active (Kunin et al, 1973; Wise et al, 1980).

Albumin is quantitatively the most important serum protein and it's effect on the disposition of acidic antibacterial agents is well established. Although alpha-1-acid glycoprotein (AAG) has a high affinity for basic drugs, concentrations of this protein in healthy animals are usually too low to affect drug disposition or efficacy. Consequently, basic drugs (e.g., erythromycin) are usually poorly protein-bound in normal animals. However, serum concentrations of AAG may increase considerably during periods of

inflammation and/or infection and this may cause an associated increase in drug binding. During these periods, when antibacterial agents are frequently used, AAG-drug interactions may be of therapeutic importance.

Although binding to serum proteins may affect disposition and tissue penetration of antibacterial agents, detrimental effects of protein binding on antibacterial activity are primarily due to drug-protein interactions at the site of infection, which is usually the interstitium. Study of these interactions is frustrated by an inability to sample sufficient quantities of interstitial fluid. Experimental infections established by inoculation of subcutaneous tissue chambers provide a convenient source of infected tissue fluid which can be subjected to in vitro analysis.

The objective of the study was to determine the effect of Pasteurella haemolytica infection on the proportion of erythromycin bound to macromolecules in serum and tissue fluid and to estimate the binding parameters describing binding between erythromycin and bovine AAG and albumin.

Materials and Methods

Animals

Six Hereford cross-bred calves 4 to 8 months of age and weighing from 100 kg to 225 kg were used in this experiment. Prior to use, the animals were dewormed with fenbendazole (5 mg/kg, Safeguard, Hoechst-Roussel) and observed on a daily basis during a two week acclimation period to establish that

they were in good health. They were maintained in small pens for the duration of the study and were fed grass and alfalfa hay supplemented with a commercial grain mixture containing 14% protein.

Implantation of Tissue Chambers

Two cup-shaped Delrin® (E.I. Du Pont Nemours) tissue chambers, measuring 46 mm ID and 15 mm in depth, were implanted subcutaneously in each calf, one chamber in each paralumbar fossa. Perforations in the walls and base of the chambers allowed unrestricted exchange of solutes and cells between tissue chamber fluid and surrounding interstitial fluids. Chamber fluid could be aspirated by inserting a needle through the skin and the silicone rubber (Silastic®, Dow Corning) membrane, which covered the top of the chamber. Tissue chamber assembly, sterilization, surgical implantation, and collection of chamber fluid samples have been described previously (Clarke et al, 1989a). The sterility of the surgical technique was monitored by culturing an aspirate of chamber fluid aerobically and anaerobically on 5% sheep blood agar.

Inoculation and Sample Collection

Approximately 2 months after implantation, preinoculation blood and chamber fluid samples were collected. Five days later, all chambers were inoculated with 1 ml of a 6 hour culture of a field isolate of P. haemolytica serotype 1 (Corstvet et al, 1973). Inocula were prepared by culture on supplemented brain heart infusion agar followed by a

suspension of the bacteria in phosphate buffered saline (PBS) at a concentration of 3 x 10^6 colony forming units/ml, as previously described (Clarke et al, 1989d). Four days after inoculation, samples of blood and tissue chamber fluid were collected and frozen at -20°C until analysis.

Dialysis Experiments

The proportion of erythromycin bound to proteins was determined by equilibrium dialysis. Plexiglass® dialysis chambers, each containing 8 compartments (200 ul capacity each side) were custom constructed. Reconstituted cellulose membranes, 0.073 mm thick and with a 6,000 M.W. cutoff (Bel-Art, Pequannock, NJ), separated protein-free and protein-containing compartments. Two hundred microliters of sample (serum or chamber fluid collected before and 4 days after inoculation) were dialyzed for 48 hrs against 200 ml 0.15 M phosphate buffer (pH 7.2) containing different concentrations of erythromycin in duplicate (0.5, 5.0 and 50.0 ug/ml). Equilibrium controls (50 ug/ml erythromycin) were included in each assay and were dialyzed for 72 hrs.

Affinity and capacity binding parameters were determined by dialyzing (48 hrs) different concentrations of erythromycin (0.5, 5, 20, 40, 60, 80, 100 ug/ml) in phosphate buffer against AAG (1 mg/ml) or albumin (3 g/l) in buffer. An equilibrium control (100 ug/ml erythromycin) was included in each assay and was dialyzed for 72 hrs.

Desired erythromycin concentrations were obtained by combining a constant amount of labelled erythromycin (0.013 ug

of 55 mCi/mmol)(New England Nuclear, Boston, MA) with varying amounts of unlabelled erythromycin (Sigma Chemical Company, St. Louis, MO). Dialysis was performed at 37°C for 48 hrs (72 hrs for equilibrium controls) in an oscillating incubator (Gallenkamp Plus No. 1, Curtin Matheson, Carrollton, TX). After dialysis, the radioactivity of 150 ul aspirated from each compartment was measured by adding 5 ml scintillation cocktail (Atomlight®, New England Nuclear, Boston, MA) and counting on a liquid scintillation counter (LS5000 TDC, Beckman Instruments, Fullerton, CA) for 5 mins. Quench correction was achieved by the automatic external standard method.

The stability of erythromycin under dialysis conditions was monitored by bioassay, using Micrococcus lutea ATCC 9341 (Bennett et al, 1966). A 5 ug/ml test standard was divided into 20 aliquots; 10 were incubated at 37°C in an oscillating incubator for 48 hours and the balance were placed in a refrigerator at 4°C for the same period. Standard concentration curves were constructed and used to assay the test standard, before and after incubation. In addition fresh test standard (5 ug/ml) was prepared and assayed concurrently with the post incubation samples.

Protein Assay

Albumin concentrations were assayed using the bromcresol green method and a commercially available chemistry analyzer. AAG concentrations were assayed using radial immunodiffusion.

Data analyses

The degree of protein binding was calculated using the following equation:

$$\% \text{ Binding} = \frac{100 \text{ X (Activity on the protein-containing side - Activity on the protein-free side}}{\text{Activity on the protein-containing side}}$$

The number of binding sites for erythromycin on AAG was determined by Scatchard analysis (Scatchard, 1948), as were the initial estimates of binding affinity constants. Binding parameters of individual data sets were then determined by using a microcomputer program (Bourne, 1989) to solve quadratic equation describing the relationship between total and free drug concentration:

$$C_{free} = \frac{(Ka*C_{tot}-1-Ka*Pt)+\sqrt{(Ka*C_{tot}-1-Ka*Pt)^2+4*Ka*C_{tot}}}{2*Ka}$$

where Ka is the binding affinity constant, Pt is the protein concentration, and C_{free} and C_{tot} are the total and free drug concentrations, respectively (Bevill et al, 1982). Minimum sums of weighted residuals ($1/C_{free}$) were approached using the Damping Gauss-Newton fitting algorithm.

The effect of P. haemolytica on binding of erythromycin to serum and chamber fluid were subjected to an analysis of variance, blocking for animals and chamber side.

Results

Binding of erythromycin to macromolecules in serum and chamber fluid ranged from 16% in noninfected chamber fluid (50 ug/ml erythromycin) to 55% in serum collected after

inoculation (0.5 ug/ml erythromycin) (Table 2 pp. 53). At two concentrations of erythromycin (0.5 and 5.0 ug/ml), binding in serum was significantly higher after inoculation compared with pre-inoculation values. Analysis of variance revealed no difference in binding to macromolecules in chamber fluids collected before and after inoculation.

Binding of erythromycin to AAG was saturable between the concentrations 0.5 to 100 ug/ml (Table 3 pp. 53) and varied between 62% at 0.5 ug/ml erythromycin and 27% at 100 ug/ml erythromycin. Scatchard analysis of data indicated a single binding site with a mean binding affinity constant of $6.45 \times 10^4 \pm 1.65 \times 10^4 M^{-1}$ (Fig. 4 pp. 58). Equilibrium analysis of the binding using the quadratic equation describing the relationship between C_{tot} and C_{free}, yielded a similar mean estimate of binding affinity ($6.91 \times 10^4 \pm 3.83 \times 10^4 M^{-1}$) at a mean protein concentration of $2.74 \times 10^{-5} \pm 6.09 \times 10^{-5}$ M (Fig. 5 pp. 59).
Erythromycin was only slightly bound to albumin (Table 3 pp. 53) and no specific binding sites could be identified by Scatchard analysis.

Discussion and Conclusions

Most infections are not limited to the blood vascular system, but are more likely to be located in the interstitium. Therefore, although vascular drug-protein interactions may affect drug disposition, interactions in tissues are most relevant to antimicrobial efficacy. Tissue cham-

bers have been validated as a model for studying interstitial infections (Clarke et al, 1989a; 1989b; 1989c; 1989d; 1992) and contain fluid that is thought to be similar to the theoretical composition of interstitial fluid. This characteristic and the ease with which these chambers can be sampled, makes them convenient models for studying interstitial drug-protein interactions. Furthermore, the chamber model allows study of protein binding in infected animals, which may be particularly appropriate when a drug interacts with acute phase proteins, such as AAG.

Protein binding at the site of infection may be affected by several factors including: temperature, pH, drug concentration, and protein concentration. Previous studies (Chapter III) indicated that inoculation of chambers caused increased concentrations of both AAG and albumin in chamber fluid and increased concentration of AAG in serum. Thus, infection is likely to affect binding of AAG to organic bases, such as erythromycin. This was confirmed by the results of the present study, in which a higher proportion of erythromycin was bound in serum after inoculation than before inoculation. Although a similar trend in binding was evident in chamber fluid, apparent differences were not large enough to be declared statistically significant. Assuming saturable binding, the effect of an increase in protein concentration has a greater effect on proportional binding when drug concentrations are relatively lower. This relationship may explain the failure to identify significant

differences in binding in chamber fluids which contain less protein and relatively more erythromycin or in serum at the highest concentration of erythromycin.

Depending on the drug concentration, erythromycin was moderately bound to AAG but only slightly bound to albumin. These data are consistent with previous reports indicating that AAG is the major binding protein for erythromycin in human serum (Dette & Knothe, 1986). Thus, erythromycin follows the same pattern as many other basic xenobiotics, in that it has a higher affinity for AAG than for albumin, which binds acidic molecules more readily (Kragh-Hansen, 1981; Dette & Knothe, 1986).

The nonlinear regression analysis served to verify the Scatchard analysis and identify the possible existence of binding sites other than those on AAG. Close correlation between binding affinity constants and protein concentrations confirmed that binding to other macromolecules was inconsequential, thus validating the dialysis methodology. Furthermore, the nonlinear relationship between C_{tot} and C_{free} confirmed the saturable nature of protein binding at the erythromycin concentrations tested.

In summary, values describing binding of bovine AAG and albumin to erythromycin approximate those reported in humans. Differences in pre- and post-inoculation binding are significant at lower and therapeutically relevant concentrations of erythromycin, probably due to increased serum concentration of AAG.

CHAPTER V

CONCLUSIONS AND SUMMARY

Inoculation of Pasteurella haemolytica into subcutaneous tissue chambers caused an increase in the AAG concentrations in both serum and tissue chamber fluids. A concomitant increase in chamber fluid albumin concentration indicated that the increase in concentration of AAG in tissue chambers was due to disruption of capillary endothelial barriers.

At lower relative concentrations of erythromycin, binding to serum macromolecules was higher in serum samples collected after infection than in those collected prior to inoculations. Erythromycin was moderately bound to AAG and only slightly bound to albumin. Scatchard analysis of data describing binding to pure bovine AAG indicated that erythromycin was bound to a single high affinity site on the protein.

A soft-tissue infection, established by inoculation of subcutaneous tissue chambers, served as a convenient model for studying the effect of infection on drug-protein interactions in interstitial fluid. Although inoculation did not cause large increases in the proportion of erythromycin bound to chamber fluid or serum proteins, small differences

may nevertheless be clinically relevant because tissue concentrations of erythromycin often barely exceed minimum inhibitory concentrations.

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APPENDIXES

APPENDIX A

TABLES

TABLE 1

MEAN (±SD) PERCENT INCREASES IN AAG AND ALBUMIN CONCENTRATIONS IN SERUM AND CHAMBER FLUID AFTER INOCULATION WITH *P. HAEMOLYTICA*

Days after inoculation	Serum AAG	Chamber Fluid AAG	Chamber Fluid Albumin
2	26.6 ± 17.5	40.9 ± 30.8	93.2 ± 66.1
4	54.9 ± 45.3	95.4 ± 39.1	122.5 ± 80.2
6	47.0 ± 22.5	103.5 ± 48.7	119.9 ± 97.4
10	30.6 ± 25.3	70.2 ± 37.4	129.8 ±136.8

TABLE 2

MEAN (±SD) PROPORTIONAL BINDING OF ERYTHROMYCIN (0.5, 5, 50 UG/ML) TO SERUM OR TISSUE CHAMBER FLUID, BEFORE AND AFTER INOCULATION OF PASTEURELLA HAEMOLYTICA

Erythromycin Conc (ug/ml)	Before inoculation	After inoculation
Serum		
0.5	0.48 ± 0.04	0.55 ± 0.02*
5	0.45 ± 0.03	0.51 ± 0.03*
50	0.27 ± 0.03	0.29 ± 0.04
Chamber Fluid		
0.5	0.34 ± 0.04	0.34 ± 0.04
5	0.32 ± 0.07	0.35 ± 0.03
50	0.16 ± 0.04	0.19 ± 0.04

* - Denotes Significant Difference

TABLE 3

MEAN (±SD) PROPORTIONAL BINDING OF ERYTHROMYCIN TO AAG AND ALBUMIN

Erythromycin Conc. (ug/ml)	AAG Percent Bound	Albumin Percent Bound
0.5	62%	15%
5	61%	14%
20	51%	13%
40	41%	11%
60	35%	9%
80	29%	8%
100	27%	7%

APPENDIX B

FIGURES

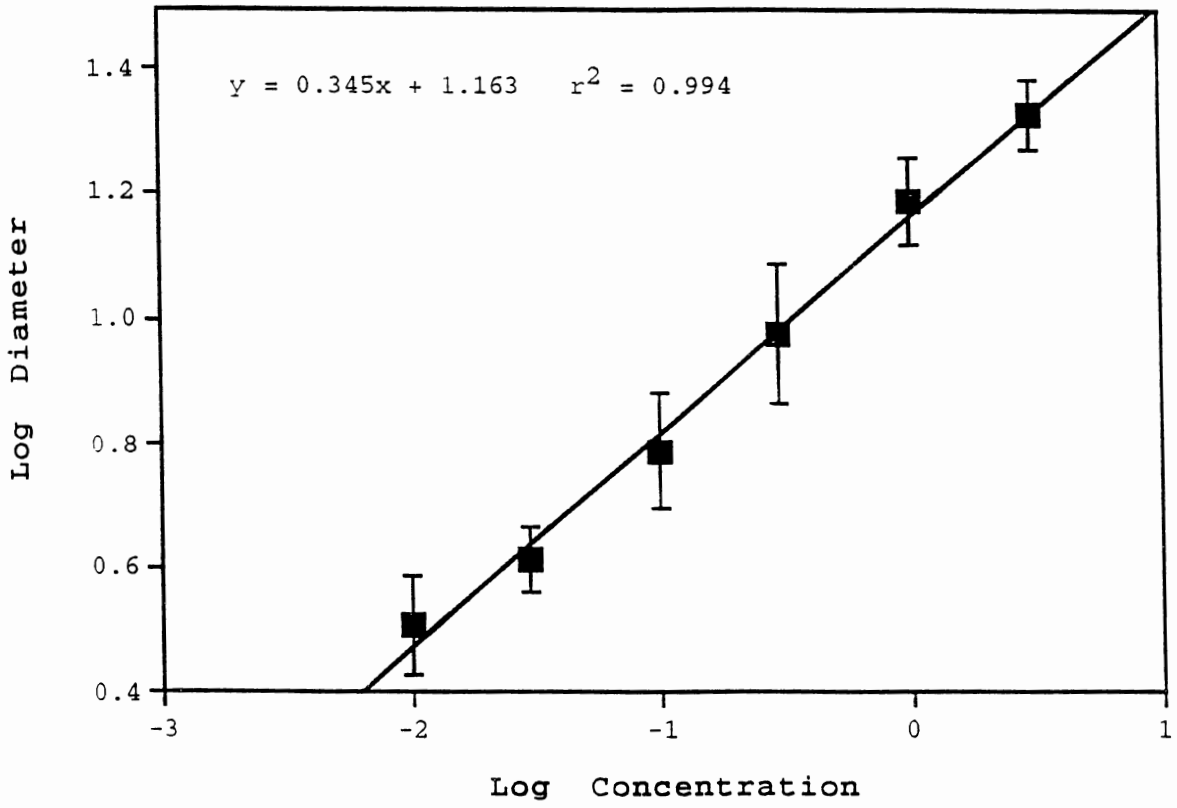

Figure 1. Mean (± SD) Logarithms of Precipitation Ring Diameters plotted against Logarithms of AAG Concentration Standards. Mean coordinates were calculated using Individual Standard Curves for each assay (gel).

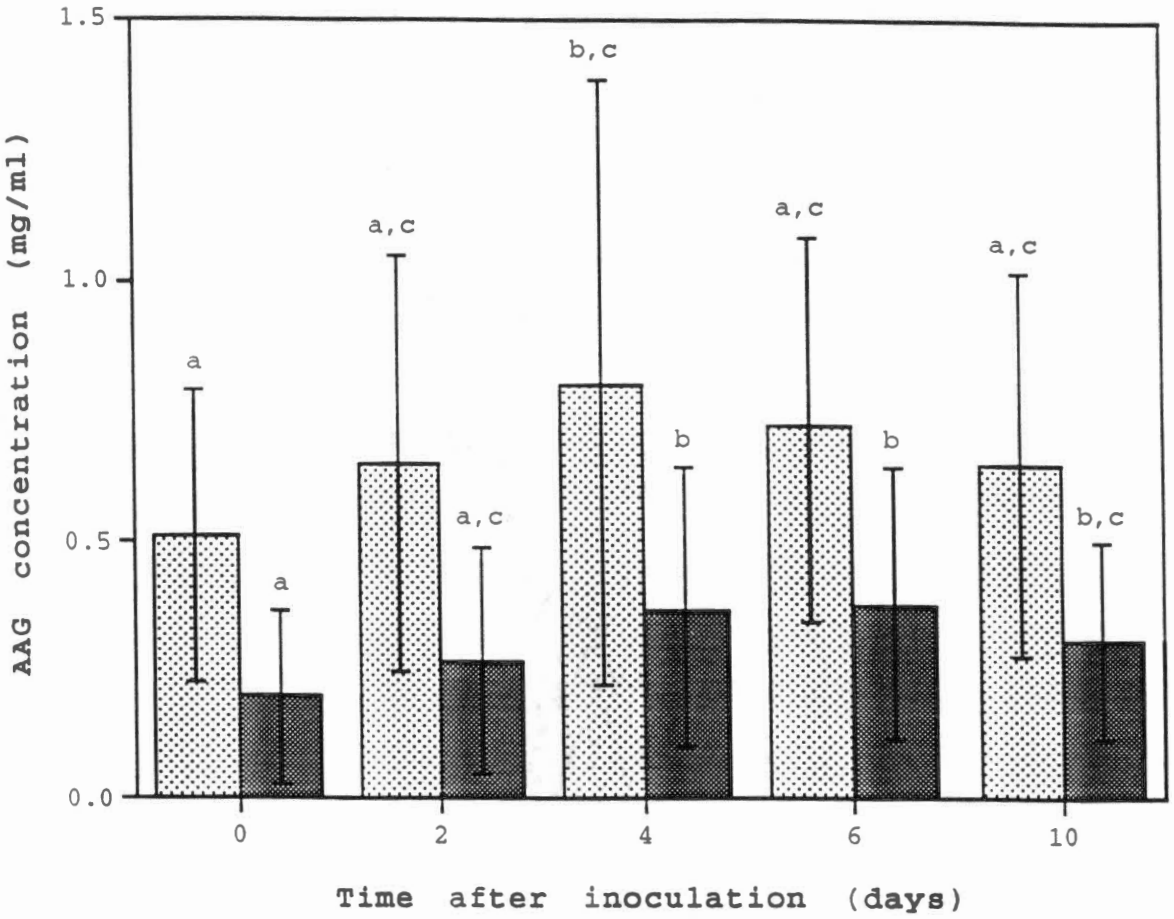

Figure 2. Mean (± SD) Concentrations of AAG in Serum and Chamber Fluid before (day 0) and after Inoculation of Tissue Chambers with P. haemolytica. For each matrix (Serum or Chamber Fluid), columns with the Same Letter are Not Significantly Different.

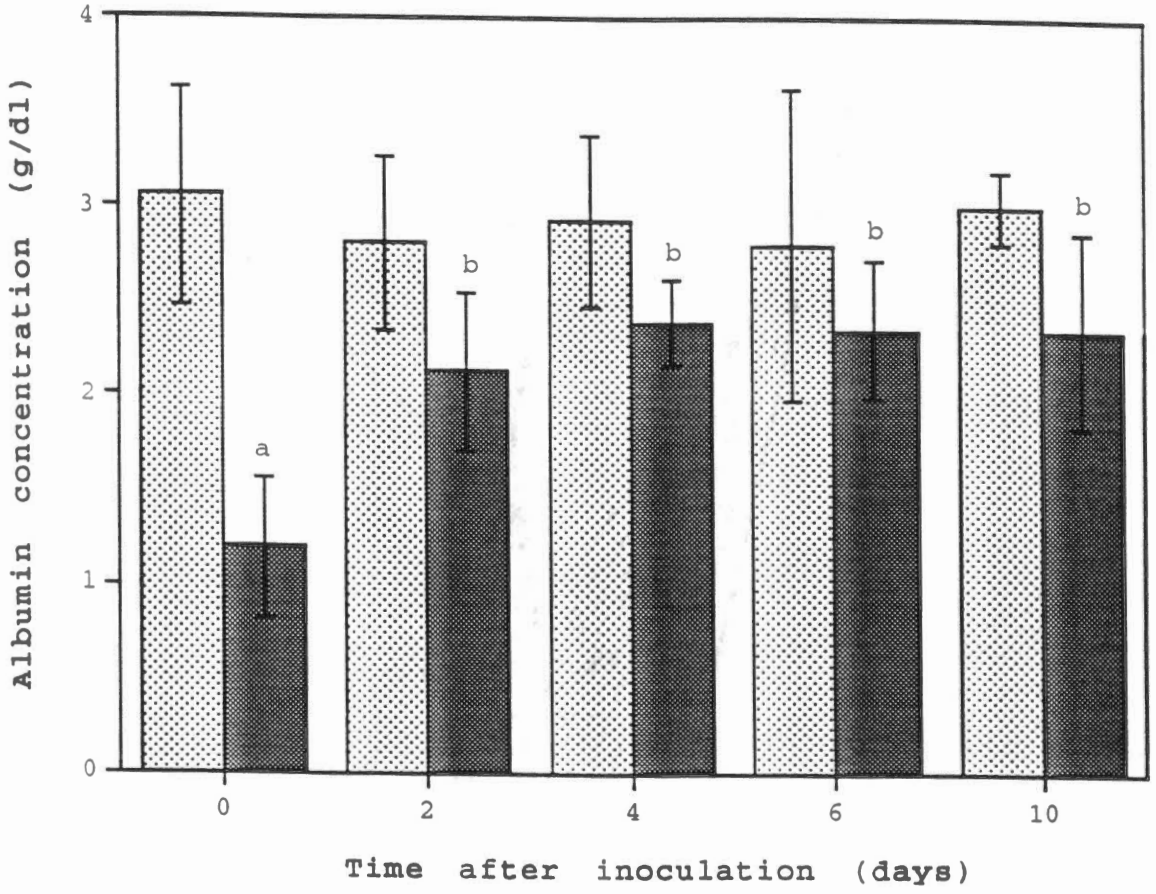

Figure 3. Mean (± SD) Concentrations of Albumin in Serum and Chamber Fluid before (Day 0) and after Inoculation of Tissue Chambers with P. haemolytica. For each matrix (Serum or Chamber fluid), columns with the Same Letter are Not Significantly Different.

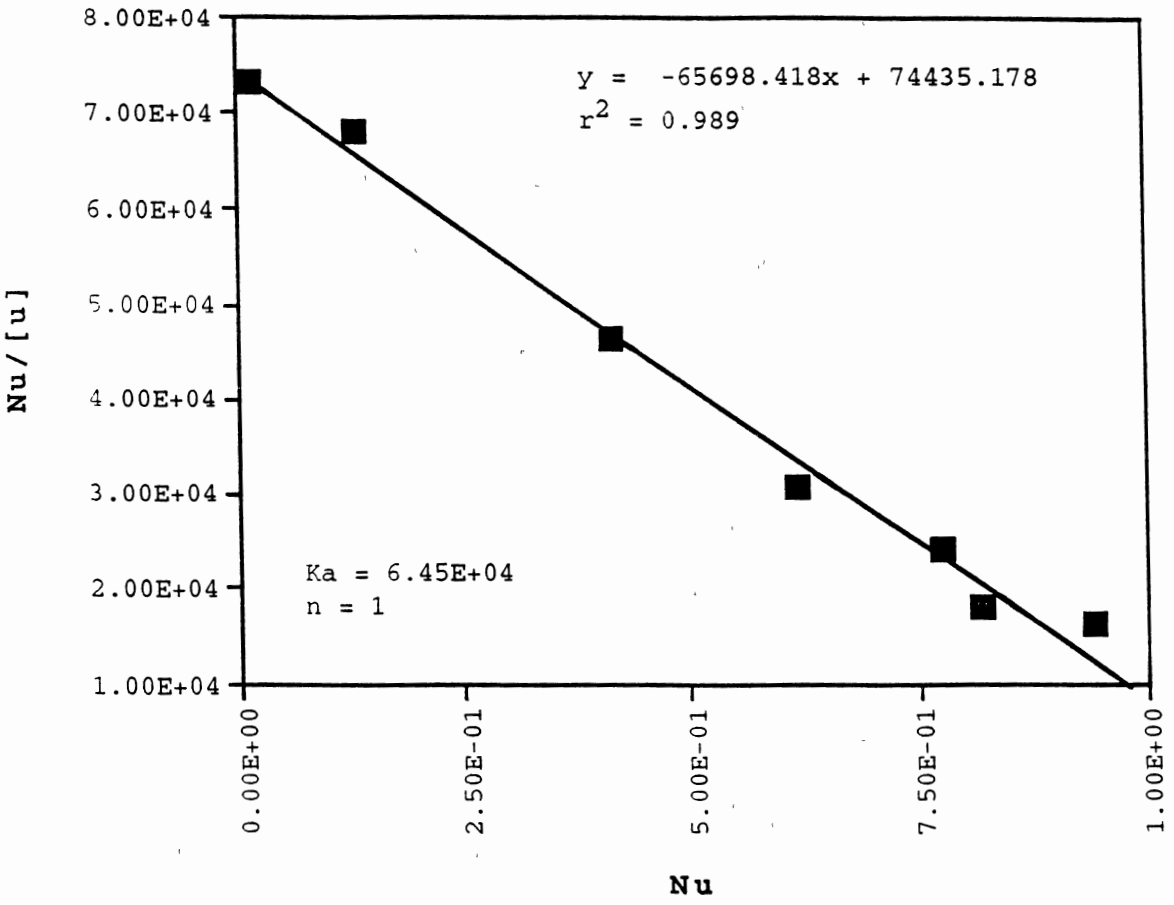

Figure 4. Scatchard Plot of Mean Data describing Binding of Erythromycin to AAG. The symbol [u] signifies the Free Concentration of Erythromycin and Nu is the Ratio of [u] to AAG Concentration.

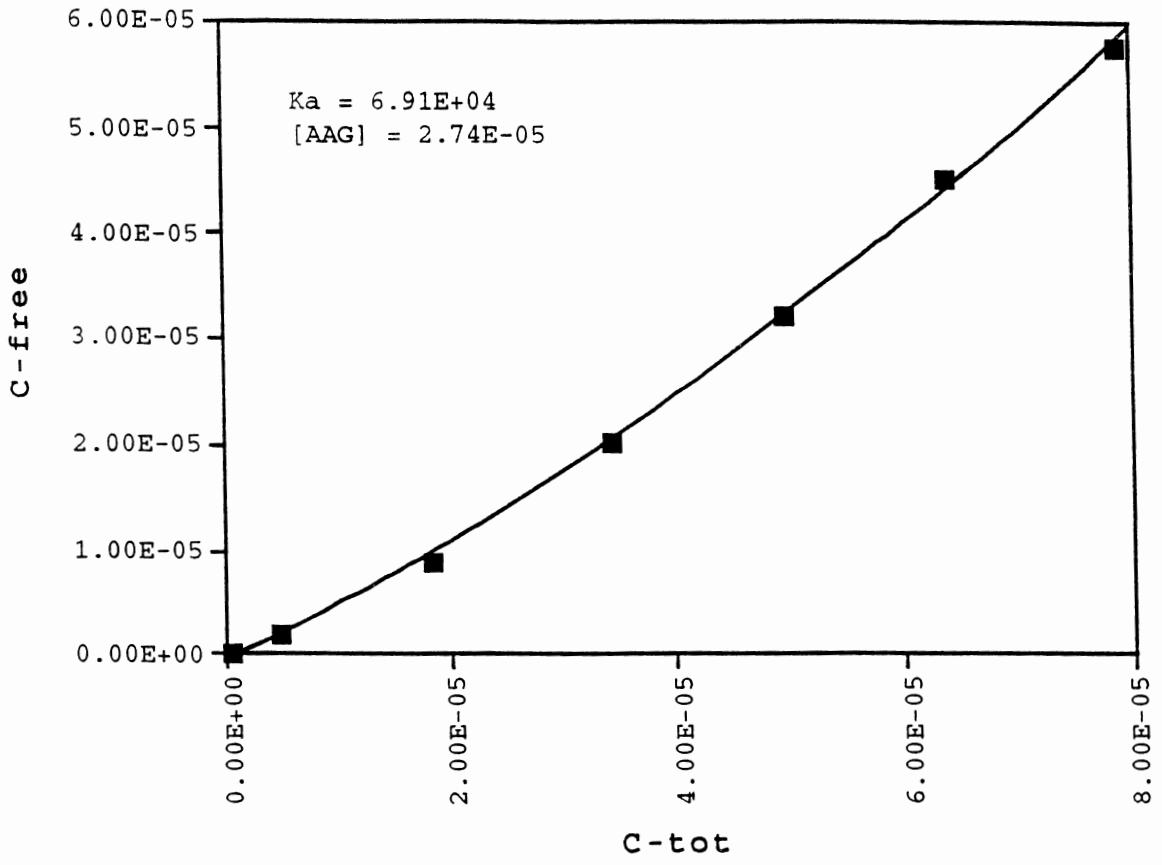

Figure 5. Quadratic Relationship between Free (C-free) and Total (C-tot) Concentrations of Erythromycin. Plot was constructed using Mean Data.

VITA

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Master of Science

Thesis: THE EFFECTS OF A LOCALIZED PASTEURELLA HAEMOLYTICA INFECTION ON THE ERYTHROMYCIN BINDING PROPERTIES AND CONCENTRATION OF BOVINE ALPHA-1-ACID GLYCOPROTEIN IN SERUM AND TISSUE CHAMBER FLUID

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