INTERACTIONS OF COLD STRESS AND *PASTEURELLA HAEMOLYTICA* IN THE PATHOGENESIS OF PNEUMONIC PASTEURELLOSIS OF CALVES

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ABSTRACT

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The objectives of this research were to identify factors of probable significance in the pathogenesis of pneumonic pasteurellosis caused by Pasteurella haemolytica in calves. Fifteen healthy calves were chilled with cold water and had a focal tracheitis induced by spraying the tracheal mucosa with a 5% acetic acid solution. Blood samples and pulmonary function tests were taken before the first stress, immediately following a second chilling and then for 12 hours subsequent to intratracheal injection with saline (control group n = 6) or saline containing 2 x 10^9 live P. haemolytica organisms (Pasteurella group n = 7). All calves were examined grossly and histologically (including 2 calves exposed to P. haemolytica but not tested for pulmonary function).

Control calves had focal areas of atelectasis and a few scattered inflammatory cell infiltrations in the lungs. These did not cause any alterations in pulmonary function tests nor in leukograms or hemograms. Cold stress in both groups of calves was associated with increased serum cortisol, O_2 consumption, CO_2 production, tidal volume, and alveolar ventilation. These alterations may facilitate pneumonia by steroid induced immunosuppression and increased exposure of alveolar surfaces to inhaled pathogens.

Alterations in pulmonary function occurred within 1 hr of Pasteurella exposure. Increases in minute ventilation were associated solely with increased dead space ventilation and by 3 hrs post inoculation decreases in dynamic compliance accompanied gas exchange impairment. The mechanism for these early changes in breathing pattern and gas exchange function remains uncertain but probably involves stimulation of intrapulmonary J receptors and local disturbances of ventilation-perfusion matching within damaged portions of lung. The data clearly indicate that the initial injury was in the lung parenchyma not in the bronchial tree. By 12 hours post inoculation calves had extensive pneumonia, with alveolar wall necrosis with edema and hemorrhage and accumulations of inflammatory cells occurring as apparently 2 independent but concurrent processes. Calves were also neutropenic and had increased serum cortisol levels. The data indicate that mast cell mediators and bradykinin do not contribute to pulmonary injury and suggests that pulmonary injury is most likely due to the combined effects of bacterial and inflammatory cell products.

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CHAPTER 1

A review of factors of possible significance in the pathogenesis of initial and developed lesions of Pasteurella haemolytica pneumonia

INTRODUCTION

Shipping Fever describes a disease syndrome of cattle and less commonly of horses and sheep which has been recognized for many years.[1-3] Because the causes and pathogenesis remain incompletely understood, this has contributed to the widespread use of "Shipping Fever" as an all-encompassing term to describe the illness.[1] The disease in cattle is a fulminating illness with high fever, anorexia, dyspnea and coughing, following a period of stress. Stress is also a term which lacks clear definition, but in the context of predisposing to Shipping Fever, stress associated with transportation is well recognized.[2] Pathologic descriptions of Shipping Fever are principally limited to those animals that become moribund or die of the disease and the lesions are typically those of severe fibrinous pneumonia[1], bronchopneumonia[2] and fibrinous pleuritis. The appearance of lungs from acutely affected animals is often described as hepatized[1-4], where the lungs become liver like in consistency and color resulting from fluid, exudate and blood accumulation in the lung parenchyma.[3-4] "Consolidation" is often used interchangeably with the term "hepatization"[4], but if used in the strictest sense implies pulmonary fibrosis and thus is incorrect for acutely developed lesions.

The difficulty with eliminating "Shipping Fever" as a problem of cattle has been due in part to the inability to identify a causative agent responsible for the lesions. Although numerous microbiologic organisms have been identified in diseased cattle affected with the clinical and pathologic changes compatible with Shipping Fever, Koch's postulates have not been fulfilled; that is, exposure of normal cattle to suspected etiologic agents fails to consistently reproduce the clinical and pathologic observations regarded as characteristic of the

naturally occurring disease. The rewards of such investigations have been to identify and classify numerous bacteria, viruses, chlamydia and mycoplasmas, that, under certain circumstances when given by themselves, may cause injury to the respiratory system of cattle.

Over the last decade, several breakthroughs in the understanding of various aspects of "Shipping Fever" have led to an alteration in the philosophy of research involving this important disease of cattle. It became apparent that Shipping Fever outbreaks were frequently associated with a variety of stressful stimuli, not simply transportation.[2] An increased understanding of natural and acquired mechanisms of resistance, particularly relating to respiratory tract disease supplemented an increasing knowledge of how stressful stimuli might alter immune responses.[5] Consequently the pursuit of a single pathogenic agent has waned and rightly so, for the disease of Shipping Fever is now seen as a complex interaction of environmental stresses, competency of host defense mechanisms and exposure to a variety of infectious agents.[1,2,6-13] Hence the terminology of bovine respiratory disease complex has largely replaced Shipping Fever in order to better accommodate these aspects of this disease.

Another important development over the last decade has been the realization of the central role Pasteurella sp. has in causing the pulmonary damage associated with Shipping Fever. The development of serologic tests for the various strains of Pasteurella and their subsequent association with many disease outbreaks of shipping fever clearly indicate that Pasteurella haemolytica serotype 1A is the principle agent involved, particularly in cases where extensive pulmonary destruction occurs.[1-3]

Pneumonic Pasteurellosis and P. haemolytica

Pasteurella sp. are not the only cause of severe pulmonary lesions associated with Shipping Fever (Bovine Respiratory Disease Complex), nor are the lesions of pasteurellosis restricted to the lungs. In cattle, a variety of bacteria may cause similar severe lesions[8,11,13,14] and bovine respiratory syncitial virus is quite pathogenic for sheep and goats as well as cattle.[15-17] In species other than ruminants, including companion animals, persons and birds, P. multocida is of greater importance than P. haemolytica. In most instances in mammals other than ruminants, lesions due to P. multocida are sporadic in nature and variable in the organs affected.[18-24] Species of Pasteurella other than P. multocida and P. haemolytica are important causes of morbidity and mortality in birds.[24] Pasteurella multocida is an important pathogen of cattle, sheep, pigs and water buffalo as a cause of pneumonia and hemorrhagic septicemia.[2,3,24-26] Pasteurella haemolytica is also an important cause of septicemia and pneumonia limited to domesticated sheep[27-33], pigs[23] and cattle[1-4,9-14] and of the various strains of P. haemolytica, only type 1A has such a profound influence on the health of cattle raised in Europe and North America.[34,35] Therefore P. haemolytica is considered preeminent as the cause of the severe, life threatening lesions of Shipping Fever, although other factors influence the initiation of pneumonia and facilitation of P. haemolytica survival in the lungs. The understanding of the importance of P. haemolytica in this regard has largely resulted from the work of Thomson and coworkers who succeeded in reproducing lesions compatible with field cases of Shipping Fever[1,7,36-43] by aerosol exposure or by direct instillation of cultures into the airways. Others have also been successful in inducing

lesions similar to field cases of Shipping Fever using P. haemolytica alone[44,45] or combined with viruses[15-17,27-29,31-33], by septic embolization of the lung[30] and using pretreatments with cold stress.[46,47] A number of investigators had limited success in inducing pneumonia with P. haemolytica[1,48-50] and there are also reports where the disease could not be experimentally induced despite administration of organisms via a number of different routes.[51-53] As stated by Rehmtulla and Thomson[1], the lesions in some of the early studies on transmission of Shipping Fever to healthy animals must be questioned, since many investigators did not investigate specific pathogens but gave inoculations of homogenized pneumonic material to healthy calves by various routes.[1,54,55] However, Carpenter and Gilman (1921) were probably successful in reproducing pasteurellosis in a calf after intratracheal challenge of an organism then known as Pasteurella boviseptica.[56] The reasons why such diverse methods of pneumonic pasteurellosis induction are successful while others have failed is not totally understood, but may relate to the concentration of organisms delivered to the lung, since most successful studies report pulmonary exposures of greater than 10^8 organisms. Such differences undoubtedly reflect the complex interactions between host defense mechanisms and the pathogenic effects of P. haemolytica.

Defense Mechanisms Against Pulmonary Pasteurellosis

Pasteurella haemolytica is considered a non-pathogenic resident of the nasal cavity of healthy cattle.[10] During breathing, aerosols containing these bacteria are generated and deposited in the lungs.[57-58] Normally cattle apparently cope with this low level bacterial exposure

and several pulmonary defense mechanisms are likely to be involved; clearance of bacteria by the mucociliary escalator[59-60]; phagocytosis by resident macrophages and migrating leukocytes[39,40,59-63]; inhibition of growth by local tissue factors[39,64]; and maintenance of suboptimal bacteria growth conditions. Tipping the balance in favor of bacterial survival is apparently not difficult in cattle. Experimental induction of disease by exposure to large numbers of micro-organisms either by direct intrapulmonary injection, or by inhalation challenge may simply overwhelm the phagocytic capacity of readily available leukocytes.[1,7,10] Viruses and irritant chemicals disturb the mucociliary escalator and thereby nullify a defense mechanism.[7,9,10,13,39,40,43] Septic embolization may place organisms in a microaerophilic environment, rich in nutrients required for growth[24] but sequestered away from efficiently functioning phagocytes. Stresses, including cold stress, may impair phagocytic responses by a variety of immunosuppressive mechanisms[5] and may facilitate growth and seeding of bacteria from the nasal cavity.[57,58,65] Experimentally induced pneumonic pasteurellosis may therefore arise by compromise of defense mechanisms in a multiplicity of ways. The relative importance of each mechanism in defending against pasteurellosis is in doubt, but Thomson and others have suggested macrophages have an important role.[1,59] Naturally occurring P. haemolytica pneumonia is facilitated by concurrent or prior virally-induced injury to the respiratory tract.[13] It has only recently been recognized that stress induced by management practices and environmental conditions is of critical importance.[5,66] Anecdotal evidence for this view is that P. haemolytica serotype 1A has a world-wide distribution yet the disease of pneumonic

pasteurellosis is confined principally to North America and parts of Europe[2], where cattle are intensively raised.

Stress As A Predisposing Factor of Pneumonic Pasteurellosis

Stress defined in the broadest sense by Seyle (1976) is that nonspecific response by the body to any demand.[67] While physical stresses (stressors) are well recognized, only recently has information regarding psychological stresses of animals become available.[5] The effects of stress on immune function of homeotherms was recently reviewed by Kelley (1980).[5] However, the effects of such environmental influences may not be restricted to the immune system. For example thermal stresses, particularly with associated alterations in humidity, predispose to respiratory disease in cattle.[5,7,10,11,48-50,55,65,67-72] Not only do these climatic conditions appear to alter the capacity of the immune system for both cell mediated and humoral responses[5] but such changes may enhance bacterial survival in the environment[65] as well as in the body and may alter the cellular structure of the airways.[72] Not all evidence suggests that such climatic changes are immunosuppressive[73], but at least for calves, Kelley suggests that evidence to the contrary is somewhat suspect.[5] Crowding, isolation, mixing of animals socially naive to one another, weaning, food deprivation, noise, restraint and excessive physical demands may lead to immunosuppression.[5] Clearly, many of the husbandry practices in cattle rearing, especially transportation of livestock for long distances, are combinations of several stressful influences.[1-10] In addition crowding, enclosure in unsanitary environments, particularly with limited ventilation, and exposure to microbial pathogens foreign to the peer group may greatly increase the load of

pathogens inhaled into the lungs, even if immune competence is maintained.[68,71,74-77]

The mechanisms involved in body responses to stress are not fully understood. Some events, such as immunosuppression appear related to the increase in corticosteroids associated with a variety of stresses, including transportation of cattle.[78,79] Corticoid related alterations in immunoglobulin absorption from the gut and corticosteroid related thymic and lymphoid atrophy are two known effects of stress in calves.[80-82] However, pharmacologic doses of hydrocortisone to calves do not prevent leukocyte migration or phagocytosis in pneumonic areas of lungs infected with P. haemolytica[39] or predispose to the development of pneumonia.[51] Although no assessment of phagocytic killing of bacteria was made in the former study[39], these data indicate that corticosteroids may not be entirely responsible for the various aspects of immune suppression summarized by Kelley.

There is no information regarding the effects of stress on other defense mechanisms in the lung[83] or on pulmonary function. Alterations in pulmonary structure and function by stressful stimuli may be a significant influence in the predisposition of stressed animals to pneumonic pasteurellosis.

Normal respiratory system structure and function and its response to injury

The prime function of the respiratory system is gas exchange. Pulmonary structure is arranged for efficient uptake of oxygen and the excretion of gaseous wastes, principally CO_2. The cessation of respiration is life threatening within minutes and so elaborate mechanisms of

control by both neural and humoral mechanisms allow prompt adjustments to maintain adequate gas exchange.[84] Since P. haemolytica pneumonia eventually leads to hypoxemia and respiratory failure, it is likely that these controlling influences are activated and/or modified by pasteurellosis. The effects of pulmonary pasteurellosis have not been determined so the importance of these events is speculative.

In cattle, other respiratory system functions of olfaction and metabolism of polypeptides by the pulmonary vasculature are less well documented, even under normal circumstances, and the effect of pasteurellosis on these functions is also unknown.

The structure of the bovine respiratory system, in health and with pasteurellosis, has received more attention than its function. The bovine lung is a completely lobulated organ so that each lobule has only one pathway to supply ventilation[85,86] (that is, a complete lack of collateral ventilatory pathways). As a result, airway obstruction in cattle may profoundly affect gas exchange[87] since no alternate pathways for gas to enter obstructed lobules exists. Other structural properties of the bovine lung may also influence function and predispose to pneumonic pasteurellosis.[88] Cattle have a relatively small gas exchange surface in relationship to their metabolic rates and minute ventilation.[88] This may not only facilitate exposure to environmental hazards but may jeopardize gas exchange with relatively minor disturbances in respiratory structure. The bovine lung has relatively few alveolar macrophages[86,89] and these are thought to require oxygen for normal function.[90,91] If terminal bronchiolar obstruction occurs in cattle lungs, the phagocytic function of macrophages, which is likely to be limited by their small numbers, will probably be further compromized by

local hypoxia.[88] Hypoxia may also depress the normal activity of mucociliary transport from these regions.[92] That these effects of hypoxia are important in calves is supported by the finding that reduced bacterial clearance rates occur in the lung regions of calves where oxygen tension is thought to be least.[63] The lobular structure of the bovine lung may also facilitate atelectasis subsequent to airway obstruction because the loose connective tissue surrounding each lobule may reduce forces of interdependence that would otherwise assist in preventing collapse of the lobule.[93] This appears to be the case for the pig[94], a species with lobulated lung similar to cattle.[85] Bovine lungs have large numbers of mast cells[86], and mediators from these cells may cause airway and vascular modifications so that gas exchange is impaired.[87]

These aspects and other functional properties (a pronounced hypoxic vasoconstrictor response[95] and an apparent lack of lysozyme in pulmonary secretions[88]) appear to place cattle in a disadvantaged position in coping with pathogens which arrive in the lung. Veit and Farrell[88] suggest that such structural disadvantages have an important role in predisposing to Shipping Fever.

The response of the respiratory system to injury clearly has a wide variety of manifestations, dependent in part on the nature of the injurious agent, and the response of host tissues. Direct effects of the injurious agent are readily appreciated with physical and thermal injuries and with the effects of viruses, and often result in cell necrosis.[13,15-17,31-33,39,40,43,96] It is not known whether P. haemolytica has any direct injurious effect on bovine respiratory epithelial cells when the bacteria remain intact. However, ample evidence exists for the release of toxic products from P. haemolytica. Pasteurella

haemolytica is a potent source of endotoxin[97], a component normally found in the bacterial cell wall and liberated upon cell destruction, and of cytotoxins.[7,97-99] In view of the known potent effects of these 2 bacterial products on leukocyte function[98,99] and of the effects of endotoxin on pulmonary vasculature[97,100-105] and pulmonary surfactant[39,106], it is likely that these products contribute significantly to functional and structural derangements that ultimately occur in animals dying of pneumonic pasteurellosis.

In addition to the effects of bacterial products, the presence of inflammatory exudate may alter lung structure and function. This exudate which develops in response to P. haemolytica, may not only destroy the bacteria but damage lung tissue by the release of phagolysozymal enzymes, biologically active lipids and mediators into the lung. This aspect is discussed in detail below.

Alterations in pulmonary structure associated with naturally occurring and experimental P. haemolytica pneumonia were reviewed by Rehmtulla and Thomson (1981)[1], who pointed out the need to determine the early lesions of P. haemolytica pneumonia, since the pathogenic processes involved in the conversion of healthy pulmonary tissue to those with pneumonia of life threatening severity are uncertain. In cases of severe pneumonia, Rehmtulla and Thomson noted discrepancies were present in the reported degree of airway involvement, of the extent of vascular thrombosis and in the nature of the inflammatory exudate.[1] Although mononuclear "swirly" or streaming cells are a characteristic of the exudate in developed cases of pneumonia, the derivation of these cells is unclear although Thomson says that they originate from macrophages.[1] Clearly, to clarify the reasons for the discrepancies in the descriptions

of the appearance of pneumonic pasteurellosis lesions, further structural studies are needed, particularly in the early stages of the disease. Only one study has addressed the lesions caused by pulmonary P. haemolytica during the early phases of infection. Gilka et al (1974)[39,40] described alveolar edema, loss of surfactant lining film and changes suggestive of increased numbers of macrophages, 4 hours after bacterial challenge by aerosol exposure. The mechanism of formation of pulmonary edema remains undetermined and as yet the processes that result in the transition of these early lesions to those of acute necrotizing and fibrinous pneumonia are unknown. Of interest, many years prior to the study of Gilka et al (1974), Edington (1930) described edema, hemorrhage and congestion of alveolar walls, with exudation of serum into alveolar lumens of field cases of pneumonia from which he consistently isolated P. boviseptica.[107] Tweed and Edington considered these changes as the early lesions but this claim was not substantiated.[107]

Host mediated responses of possible importance in the pathogenesis of P. haemolytica pneumonia.

a. Hormonal influences

I. Corticosteroids. As previously described, corticosteroids are probably liberated in response to stressful stimuli, even in neonatal calves.[5,78,79,108] Disease itself may pose a stress which results in further steroid release, as evidenced by increased corticoid levels of young calves with diarrhea.[109] It is not known whether P. haemolytica pneumonia stimulates the

release of corticosteroids, nor is it apparent from previous studies whether such a response is likely to be beneficial or detrimental to the animal. Steroids may aid in stabilization of cell membranes in response to injury[110], may reduce pulmonary edema, improve ingested immunoglobulin uptake[5,80,81], stimulate surfactant[111] and may enhance interferon production.[112] However, immunosuppression[5,78,79,82] and possible detrimental effects on pulmonary alveolar type II cells[39,40], pulmonary growth[111] and histamine metabolism[113] may be of greater importance than the above beneficial effects. Evidence regarding the detrimental effects of corticoid in calf lungs is conflicting[39,40,51] even when given in pharmacological doses.

II. Thyroid hormones. Thyroid hormones may play a role in the host responses to P. haemolytica for the following reasons. The hypothyroid state in human neonates increases the incidence of pulmonary disease[114] and small alterations in thyroid hormone levels appear to indirectly affect histamine metabolism.[113] Such a "permissive effect" may be a nonspecific action of thyroid hormone since thyroid hormones appear to exert such a "permissive" effect on the actions of other hormones for a wide range of host tissues[115]; there is a seasonal incidence of pneumonia, illness in calves being most common in winter and spring.[69,70,74] Although it appears likely that the predisposition to pneumonia is based on rather abrupt changes in climatic conditions[69,70], more rapid than that possible for changes in thyroid hormone to normally occur,[115] the influence of seasonal changes in thyroid hormone levels and the predisposition to

pneumonia remain to be proved. Since P. haemolytica is unlikely to directly affect thyroid function, it also seems unlikely that deficiency of thyroid hormones contributes to the disease because reduced thyroid hormone levels occur at the time of year when the incidence of Shipping Fever is least.[69,70,116]

III. Other hormones. The lungs are influenced by a variety of other hormones. Stress, particularly that associated with fear, excitement or cold, probably exposes the lung to increased amounts of catecholamines liberated from the adrenal medulla. If influenza and parainfluenza viral infections alter the responsiveness of the lung and leukocytes to cholinergic and adrenergic stimuli, as they are reported to do in guinea pigs[117] and persons[118-119], catecholamines may have modified and perhaps adverse effects in calves developing pasteurellosis with concurrent or previous Parainfluenza 3 virus exposure.

It is unclear whether the sex hormones have any influence on the development of Pasteurella pneumonia. Pretreatment with estrogens did not facilitate the development of pneumonia in cattle[51], but the success rate in experimental pneumonia induction was very poor exclusive of estrogen pretreatment, in this study sex differences that exist with other diseases and possibly with pasteurellosis may relate to different social pressures placed upon male and female animals, rather than a direct hormonal effect.[5]

b. Humoral Factors

I. Bradykinin. Bradykinin may be a mediator of pulmonary damage in cattle, as has been suggested by studies of bovine

anaphylaxis.[120-122] Studies *in vivo* indicate that the effects of bradykinin are most likely limited to the vasculature as intravenous and aerosol bradykinin exposure of calves does not result in alteration of pulmonary mechanical or gas exchange properties.[123] In studies where ventilation is maintained during bradykinin administration, bradykinin causes systemic and pulmonary hypotension[123]; in other studies where normoxia may not have been maintained, pulmonary vasoconstriction was attributed to bradykinin.[121,122,124-127]

The generation of bradykinin within the vasculature results from activation of plasma kallikrein, an enzyme which cleaves bradykinin, a nonapeptide, from circulating proteins called kininogens.[128,129] Increased intravascular levels of bradykinin are generally described to cause smooth muscle contraction, vasodilation, increased vascular permiability, pain and the chemotaxis and modulation of leukocyte responses[129-132] and so have been thought to contribute to the vascular damage, leukocyte infiltration and pulmonary edema associated with bovine anaphylaxis. In our research studies where ventilation was controlled, bradykinin administration did not cause pulmonary edema.[123] Since respiratory acidosis may activate mechanisms of bradykinin generation[133] and since hypoxemia limits bradykinin breakdown[134] (a normal function of angiotensin converting enzyme found bound to the pulmonary vascular endothelium[134,135]) the detrimental effects of pasteurellosis on gas exchange and vascular integrity may lead to the accummulation of bradykinin in the lungs and cause pulmonary edema and inflammation. In addition,

P. haemolytica may release endotoxin that activates Hageman factor[136,137], and activated Hageman factor is a potent promoter of bradykinin formation.[128,129,138] *Pasteurella* sp. could also activate bradykinin by pathways independent of Hageman factor, by liberation of plasminogens, kallikreins and the direct effect of endotoxin on leukocytes which ultimately leads to leukocyte secretion of kallikreins.[137]

II. Complement. Endotoxin, Hageman factor activation, and bradykinin activation all lead to the stimulation of the complement enzyme cascade system ultimately causing the generation of C3 and C5 fragments which are potent chemotactic factors for neutrophils and also result in pulmonary congestion, hemorrhage and edema when injected in lungs.[137,139-141] It is not known whether complement fragments are generated during *P. haemolytica* pneumonia of cattle, and if so what effects they have on bovine lung structure and function.

III. Hageman factor and components of the clotting system. *Pasteurella haemolytica* may cause endothelial damage via the effects of endotoxin and cause blood coagulation because of exposure of the vascular clotting factors to tissue thromboplastins.[135-138,142,143] In addition, as previously mentioned, *Pasteurella haemolytica* may activate Hageman factor by both endotoxin-dependent and endotoxin-independent mechanisms leading to stimulation of blood coagulation by the intrinsic clotting pathway.[136,137,142,143] In calves, as in other species endotoxemia may result in disseminated intravascular coagulopathies, in part due to activation of blood coagulation and in

part due to platelet aggregation.[100-106,144-146] Although such pathogenic mechanisms may explain the development of large areas of coagulation necrosis in well developed lesions of Pasteurella pneumonia due to vascular thrombosis and infarction, it is not clear why vascular thrombosis is not a consistently reported lesion of pneumonic pasteurellosis.[1]

IV. Histamine. Circulating histamine may arise from several sources. The major proportion of body histamine stores resides in mast cells[130,135], with lesser contributions from non-mast cell sources such as platelets and circulating blood granulocytes, and from the intestinal tract.[130,145-148] Endotoxin may trigger platelet[149] and granulocyte degranulations[150-152] and certain viral[153] and bacterial infections[154,156] also appear able to cause increased histamine secretion as well as increased pulmonary sensitivity to histamine.[154] Complement fragments may also cause mast cell degranulation.[139,140] Histamine has bronchoconstrictive and hypotensive properties in calves[87] and if it is liberated in significant amounts during Pasteurella infection it is likely to contribute adversely to pulmonary function.

V. Immunoglobulins. Immunity to P. haemolytica can be stimulated and circulating immunoglobulins appear to be important in establishment of immunity[37,157], although other studies have emphasized the role of the pulmonary macrophage.[44,45,64] Immunity to P. haemolytica endotoxin prevents pneumonic pasteurellosis[103,158-160], but this is not surprising since endotoxin is part of the bacterial cell wall and may act simply as a surface antigen for immunoglobulin attachment.[103]

Stimulation of humoral immunity may not be without adverse consequences, as vaccination under certain circumstances (aerosol inhalation challenge with adjuvants combined with subcutaneous vaccination) appears to make the disease worse.[36] It is not known whether circulating immunoglobulins contributed to the development or retardation of lesions in naturally occurring cases of P. haemolytica pneumonia or in the above study.[36] Certainly, immune mediated pulmonary diseases are well recognized in other species, but to date there is no evidence to indicate that a specific syndrome exists which is associated with P. haemolytica infection.

VI. Other humoral factors. Through the effects of endotoxin, and perhaps by other mechanisms, platelets, mast cells and circulating leukocytes may liberate a variety of substances that circulate in the blood. These may include ADP, serotonin, dopamine, prostaglandins and leukotrienes, and in addition, fragments of fibrin degradation products, complement and kinin fragments.[128-138,142-146,149-151] There are complex interrelationships between the activities of many of these compounds on the tissues they effect. The importance of these factors in the pathogenesis of pasteurellosis has not been evaluated.

c. Cellular factors

I. Platelets. Platelets may become involved in pneumonic pasteurellosis through a variety of mechanisms. Vascular damage, the disturbance of endothelial prostaglandin synthesis, the presence of vasoactive agents such as histamine, bradykinin activated Hageman factor, clotting and complement fragments and

the effects of endotoxins and cytotoxins from Pasteurella haemolytica may cause platelet thrombus formation and the further release of vasoactive agents via platelet degranulation.[129,130,135-138,144-146,149-152,160,161] Fibrinous thrombosis of the pulmonary lymphatics is one of the hallmarks of well developed lesions of pneumonic pasteurellosis[1-3], yet vascular thrombosis is not a consistent finding. Therefore, the contribution of platelets to the pathogenesis of Pasteurellosis is in doubt. In cases where vascular thrombosis is a feature of the disease, it would seem likely that platelets are involved in some way.

II. Neutrophils. The role of neutrophils in the development of pneumonic pasteurellosis is in doubt. Structural studies of well developed pulmonary lesions do not consistently describe an inflammatory response dominated by neutrophils and some investigators claim that purulent exudate which consists mainly of neutrophils is atypical for pneumonic pasteurellosis.[1] Furthermore, there is no evidence to date of the functional effects of neutrophil aggregation in the lungs apart from the physical effects of airway obstruction with exudates.

It seems unlikely that neutrophils are excluded from the inflammatory response to P. haemolytica in the bovine lung, since in other species, Pasteurella sp. elicit a suppurative inflammatory response.[3,18-22] A special circumstance may arise, however, when inhaled bacteria are readily cleared by normal pulmonary defense mechanisms.[39,59,60,62] With bacterial persistence, it does seem likely that neutrophils would be recruited.[62] Several previously discussed mechanisms may serve

to recruit neutrophils. Antigens including endotoxin are chemotactic for neutrophils and in vivo endotoxin causes sequestration of neutrophils within pulmonary capillary beds.[102,137,144,146,150-152] Additional factors released from macrophages[162], plus the possible effects of kinin[129-132], complement[139-141] and fibrin fragments may serve to heighten chemotaxis and lead to further accumulations of neutrophils within the lungs. Obviously, such a response would optimistically lead to the destruction of the bacteria and prevent further pulmonary damage. All too frequently the outcome is lethal to the animal and there is no compelling evidence to suggest that a heightened neutrophilic response and subsequent infiltration of the lung is beneficial to the animal. Indeed, the possibility exists that through cell degeneration and lysis, perhaps facilitated by the bacterial release of endotoxin and cytotoxin[97-106], neutrophils may further damage the lung through the release of a variety of chemicals, including leukotrienes and hydroperoxy fatty acids, neutral proteases and a variety of oxidative radicles including hydroxyl, hydrogen peroxide and superoxide anions.[163-166] Evidence in support of the potential for neutrophils to exacerbate tissue injury in the lungs was demonstrated in sheep, where neutrophil depletion prevents the pulmonary edema otherwise associated with endotoxin injections.[167] In addition neutrophils and their products cause tissue inflammation in organs other than lungs when injected in the tissues of cattle.[165]

III. Mononuclear cells. There is no doubt that mononuclear cells are involved in the response to P. haemolytica pneumonia. Alveolar macrophages are the first line of defense, and represent an important mechanism of bacterial clearance from the lungs.[1,39,59-62,64] Lymphocytes and plasma cells are responsible for immunity which under certain circumstances prevents the disease.[37,157] Aside from phagocytic properties macrophages can secrete a large array of substances, including enzymes, complement components, enzyme inhibitors, nucleotides, endogenous pyrogen, oxygen radicles, bioactive lipids, chemotactic factors and factors controlling the rate of replication and function of other inflammatory cells.[162] As with neutrophils, some of these secretory products have the ability under certain circumstances to cause tissue injury.[162,168,169] Another interesting aspect of the involvement of lymphomononuclear cells in pneumonic pasteurellosis is that these cells probably are the source for the so called "swirly" or "streaming" cells characteristically seen in developed lesions of P. haemolytica pneumonia. These cells appear to be degenerating mononuclear cells which have basophilic smudged nuclei and basophilic streaming cytoplasm to form fusiform shapes with frequently no clear limiting membrane. This reaction appears peculiar to pasteurellosis in cattle[1,3] and so the discovery of the mechanisms which lead to the formation of "swirly cells" may have important implications with regard to understanding the pathogenesis of pasteurellosis.

IV. Mast cells. In the intact animal, mast cell degranulation may be effected by a wide variety of entities, including immunoglobulins, principally IgE and IgG_4[169-174], complement fragments[139,140], proteolytic enzymes[175,176], phospholipids[177], prostaglandins[178], catecholamines[179], kinins, serotonin[180], and hypoxia.[181] It is not known whether mast cell degranulation occurs as a result of exposure of cattle to P. haemolytica, but there would seem to be ample opportunity for this to occur.

V. Alveolar epithelial cells. Membranous (type I) alveolar epithelial cells are metabolically active cells which may be sensitive to noxious stimuli such as hypoxia, bacterial products or products from inflammatory cells. These cells are united by tight junctions so that the alveolar epithelial barrier normally is extremely competent at preventing fluid loss from the interstitium into the alveoli.[182] Under conditions of vascular damage and lymphatic thrombosis, compromise of the barrier occurs and may explain the formation of alveolar edema in cases of pasteurellosis. However, the propensity for alveolar fluid formation may be greatly exacerbated if P. haemolytica had specific cytotoxic effects on membranous alveolar cells. Developed lesions of pneumonic pasteurellosis often show extensive necrosis of alveolar walls[1] suggesting that by some mechanism, membranous alveolar cells are damaged.

Cuboidal (type II) alveolar cells are primarily responsible for surfactant production in the lung. Gilka et al (1974) reported that some of the earliest lesions of pasteurellosis in calves (4 hrs after aerosol challenge) were related to pulmonary

edema and the loss of surfactant from the lungs with subsequent atelectasis.[39,40] In addition it was noticed that P. haemolytica organisms were frequently deposited on the surface of type II alveolar cells.[40] Whether this simply represents physical trapping of bacteria against the microvilli of these cells compared to the smooth surfaces of type I alveolar cells or was due to a specific adherence of bacteria to these cells is unknown. Furthermore, type II alveolar cells appear to be of considerable importance immunologically, at least in the stimulation of alveolar phagocytes after viral infections[183], and alveolar type II cell-macrophage membrane connections have been observed in calves (Slocombe and Echt, unpublished observations). Therefore, importance of alveolar type II cells in the pathogenesis of pasteurellosis appears likely, both through alterations in surfactant production, by the possible toxic effects of the bacteria on the cells[106] and by increased surfactant requirements as a result of alveolar edema, and for the type II cell's possible role in the immune response against P. haemolytica.

VI. Airway epithelial cells. Although many of the structural characteristics of the airway epithelial types are well described[184-189], alterations in function of these cells in response to injury[190-196], and the subsequent effects of their alterations on respiratory function are poorly understood. Influences such as hypoxia[196], exposure to noxious gases, cholinergic stimulation and the presence of mediators may affect the function of ciliated, mucogenic (goblet), serous, Clara and APUD (amine precursor uptake and decarboxylation) cells[191-195]

and result in alterations in mucociliary clearance and neural control of airway caliber. Necrosis of airways and in some cases hyperplastic epithelial responses are reported in pasteurellosis[1], but whether these changes contribute to the pathogenesis of pneumonic pasteurellosis is unknown.

VII. Pulmonary connective tissue. Although pulmonary connective tissues have received considerable attention and were recently reviewed (Hance and Crystal, 1975)[197], the mechanisms for responses of connective tissues in the lung to infectious processes is not known. Secretions from macrophages, including acid hydrolases, glycosidases and proteases may damage the connective tissue.[162,168,169] Macrophage involvement is extensive in the well developed lesions of P. haemolytica[1,3] when irregular necrotizing foci are commonly observed. These foci often extend between lobules with no apparent confinement by the lobular connective tissue.[1,3] Perhaps macrophage products are involved in the development of the connective tissue necrosis in these lesions.

Experimental Rationale

Shipping Fever remains an economically important disease of North American cattle.

The pathogenesis of Shipping Fever remains poorly understood, although the etiologic agent Pasteurella haemolytica is now known to have a central role in the rapid destruction of pulmonary tissue of cattle suffering from the disease. In addition, although a variety of stressors have been identified which predispose to Shipping Fever, the

mechanisms by which this predisposition develops are incompletely understood. Shipping Fever lesions associated with pneumonic pasteurellosis represent a well defined disease in terms of the pathologic state of the lungs at the time the animal becomes moribund or dies from the disease. However, descriptions of the early lesions of P. haemolytica pneumonia are inconsistent with the lesions that occur later in the course of the illness, and the extent of current knowledge regarding the mechanisms of pulmonary injury by P. haemolytica limited by a lack of information on the changes that occur with the initial injury to the lungs.

For these reasons, an experimental series was designed to address the functional and structural changes that occur in bovine lungs following a known important stressor (cold stress by chilling) alone, and in combination with experimentally induced P. haemolytica pneumonia. A variety of hormonal, humoral and hematologic variables were measured in order to identify factors of possible significance in establishing pulmonary injury. Because of the many humoral factors that could possibly contribute to the disease, two were selected to represent sources of activation. Bradykinin was measured as an intravascular "trace" because its activation is coupled with the clotting, plasmin and complement cascades. Histamine was chosen to represent stored mediators released by mast cells. Previously, the effects of both these mediators on lung function had been determined.

To monitor pulmonary function gas exchange and pulmonary mechanical properties were measured. The latter were measured in order to localize the functional site of injury. Briefly, dynamic compliance measurements reflect pulmonary elastic properties and small airway caliber. The

status of large airways is measured by determination of airway resistance. Because the stage of lung inflation can influence these measurements, it was necessary to determine lung volume at the point of measurement of resistance and compliance. This was achieved by the determination of functional residual capacity by Helium dilution.

Pulmonary structural changes were determined using routine histopathologic techniques, and structural findings correlated with the hematologic and pulmonary functional studies. In order to control the state of pulmonary inflation in structural studies, lung samples were fixed by airway perfusion under a constant pressure.

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CHAPTER 2

Pathogenesis of bovine pneumonia caused by Pasteurella haemolytica: method of induction and changes in the circulating blood constituents during the onset of pulmonary injury

SUMMARY

Six healthy neonatal calves were chilled with cold water and had a focal tracheitis induced by spraying of 5% acetic acid into the tracheal lumen. The effects of these stresses on total and differentiate white cell counts; plasma cortisol, thyroxine, triiodothyronine, histamine and bradykinin; hematocrit, total plasma solids and indices of the erythrocyte size and hemoglobin content were determined over the subsequent 12 hrs. Cold stress increased plasma cortisol values for less than 1 hr, but did not alter any other variable. This group of calves served as a control group for a second series of neonatal calves (n = 7) which received 2 x 10^9 organisms of P. haemolytica intratracheally immediately following an identical period of chilling and 5% acetic acid exposure. Calves receiving P. haemolytica became neutropenic. There was a trend toward increased numbers of circulating band neutrophils by 12 hrs post exposure, and plasma cortisol levels were maintained at the same or greater than cold stress levels for all measurement periods subsequent to exposure. Contrary to previous reports, this data suggests a role for the neutrophil in the pathogenesis of early lesions of pasteurellosis. While the association of corticosteroid release with stress and subsequent infection is clear, our data provide no evidence to support the contention that histamine or bradykinin are involved in the pathogenesis of the acute lesions of Pasteurella pneumonia.

INTRODUCTION

Although Pasteurella species have been incriminated in Bovine Respiratory Disease complex (BRD) for many years, the mechanisms by which they induce pulmonary injury and the pathophysiology of pneumonic

pasteurellosis have not been investigated. In this series of papers we investigate changes in the circulating blood and the lungs during the onset of experimental P. haemolytica pneumonia.

A variety of methods are used to induce pneumonic pasteurellosis in cattle.[1] In normal animals, instillation of viable bacteria into the lungs generally results in rapid clearance and fails to produce lesions typical for BRD associated with P. haemolytica.[2-5] The method we used was initially described by Breeze and Magonigle (1976)[6] and does not require the prior exposure of the respiratory tract to viruses, yet produces lesions that are very similar to spontaneously occurring pasteurellosis. Although this method, which utilizes a brief exposure to cold stress before pathogen exposure, is well documented as a successful method for induction of P. haemolytica pneumonia, the specific effects of cold stress on the respiratory system that may influence establishment of bacterial colonization are not understood. In this report we describe the methods used to initiate Pasteurella pneumonia in calves, and identify alterations in circulating blood constituents including plasma, bradykinin and histamine, serum cortisol, Triiodothyronine (T_3) and thyroxine (T_4), total solids, hematocrit, the leukogram and hemogram, which result from cold stress and from intratracheal inoculation of P. haemolytica.

MATERIALS AND METHODS

Studies were performed on 13 clinically healthy, male neonatal Holstein calves obtained from a local dairy. Calves were deeply sedated

with xylazine[a] (0.1 mg/kg IV) and transported to the MSU Veterinary Hospital where they were allowed to recover. Care was taken to avoid either heat or cold stress to the animals during the transportation process and during recovery from sedation. Each calf was anesthetized for placement of catheters in the carotid artery and external jugular vein and to make a tracheostomy incision. Anesthesia was induced with halothane[b] using a face mask and once the tracheostomy incision was made and endotracheal tube placed, anesthesia was maintained with a halothane-oxygen mixture using an in-circle system.

Four hours after recovery from anesthesia, baseline data were collected as outlined below. Following baseline data collection, calves were then stressed by hosing with cold water (approx. 11 C) for 20 minutes on two occasions, 12 hrs apart. In addition, each calf was given 0.5 ml of a 5% acetic acid solution in sterile saline, delivered as a spray to the tracheal mucosa a few centimeters distal to the tracheostomy site. The spray was given twice, immediately following each period of cooling.

All variables were measured immediately following the second period of chilling with water and exposure of the trachea to acetic acid. This measurement period was designated "stress".

Calves were then exposed to 20 ml of sterile saline injection (control group n = 6) or to 20 ml of P. haemolytica broth consisting of a suspension of 10^8 organisms/ml in saline (Pasteurella group n = 7). Instillation was performed rapidly via a large bore catheter and was

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a Rompun, Haver Lockhart, Shawnee Mission, Kan.
b Halothane, Halocarbon Laboratories Inc, Hackensack, NJ.

begun at the onset of inspiration. All variables were measured immediately following instillation of saline (control group) or of the P. haemolytica suspension intratracheally and this measurement period designated as T = 0. Measurements were repeated at 1, 2, 3, 6 and 12 hrs after tracheal instillation.

The P. haemolytica was obtained from a field isolate of BRD[c] and was of serotype 1A.[d] The original isolate was supplied as a pure culture in ovine liver and grown in enrichment broth[e] for 12 hrs at 37 C. A 1 ml aliquot of this broth was transferred to a second similar broth and incubated for 6 hrs at 37 C. This second inoculum, totally 50 ml, was centrifuged and washed twice in saline before resuspension in saline. The concentration of 10^8 organisms/ml was achieved by matching the optical density of the culture to that of known standard bacterial suspensions. Viability of the organisms was confirmed by a quantitative plate count.

At each measurement period, blood was withdrawn into siliconized syringes from siliconized[f] polyethylene[g] catheters placed in the jugular vein and carotid artery. Samples of arterial blood were placed in glass tubes containing sodium EDTA[h] for the determination of total and differential white cell counts, erythrocyte count, hemoglobin content,

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c The isolate was supplied by Dr. C. Smith, Ohio Agricultural Research and Development Center, Dept. of Veterinary Science, Wooster, Ohio.
d Typing of the isolate was by Dr. C. Smith, Ohio Agricultural Research and Development Center, Dept. of Veterinary Science, Wooster, Ohio.
e Dehydrated BHI, Difco Laboratories, Detroit, Mi.
f Siliclad, Clay Adams Division, Becton Dickinson and Co, Parsippany, NJ.
g PE tubing, Clay Adams Division, Becton Dickinson and Co, Parsippany, NJ.
h EDTA vacutainer, Becton-Dickinson Division, Becton Dickinson and Co, Rutherford, NJ.

packed cell volume and plasma total solids. Characteristics of the erythrocytes (size and hemoglobin content) were determined using a Coulter counter.[i]

Bradykinin Assay. Paired arterial and venous samples were collected into chilled 10 ml polypropylene tubes which had been previously siliconized and which contained 3.6 mg of hexadimethrine[j] and 9.0 mg of sodium EDTA.[k] Each sample was gently mixed, then centrifuged at 2600 rpm using a chilled centrifuge.[l] A 5 ml aliquot of plasma from each sample was precipitated with 0.25 ml of 20% trichloroacetic acid.[m] After centrifugation, the supernatant and a single rinsed supernatant from the original precipitated pellet were combined and rapidly frozen. These were subsequently analyzed for bradykinin content by radioimmunoassay.[7,n]

Briefly, the thawed supernatant was added to an Amberlite CG-50 column, washed with 0.1 N acetic acid and bradykinin eluted with 50% acetic acid. The eluate containing bradykinin was freeze dried and then resuspended in sodium barbital buffer. A 16% solution of polyethylene glycol[o] was used to separate from free bradykinin. ^{125}I labelled bradykinin was used to validate the assay system (recoveries in the initial study were from 90% to 105%, with intra-assay coefficients of variability of $\pm$ 10% and inter-assay coefficients of variance of $\pm$ 18%).

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i Coulter counter Models SSR and MHR, Coulter Electronics Inc, Hialeah, Fla.
j Polybrene, Polysciences, Inc, Warrington, Penn.
k Sodium EDTA, Mallinckrodt, Inc, Paris, Ky.
l Model PR-6 Refrigerated centrifuge, International Equipment Co, Needam Heights, Mass.
m Trichloroacetic acid, Mallinckrodt, Inc, Paris, Ky.
n Radioimmunoassay for bradykinin was performed in the laboratory of Dr. G. Williams, Brigham Young Hospital, Boston, Mass.
o Carbowax 6000, Union Carbide Inc.

Histamine Assay. Paired arterial and venous samples were collected into chilled, heparin coated glass test tubes.[p] Samples were centrifuged at 2600 rmp for 10 minutes and the plasma separated and rapidly frozen. Histamine content was determined by fluorometric assay.[8,q] The thawed samples were dialyzed against 30% NaCl and the histamine extracted after reacting with NaOH containing 1 x 10^{-3}M EDTA and N-butanol. The histamine, still in aqueous phase was acidified with 0.1 N HCl and N-heptane added. The aqueous phase, again containing histamine, was extracted, alkalinized and O-phthalaldehyde (OPT) reacted with histamine. After a suitable period for reaction had elapsed the reaction was stopped by the addition of 0.73 M phosphoric acid and the amount of histamine bound to OPT determined with a fluoronephlometer with exciting filter peak transmission at 350 nm.[r] Sensitivity of the assay was in the order of 100 pg/ml.

Assay for Thyroxine (T_4), Triiodothyronine (T_3) and Cortisol. Paired arterial and venous blood samples were collected into glass test tubes and allowed to clot. The samples were centrifuged and the serum collected and frozen for subsequent analysis of T_3, T_4 and cortisol by radioimmunoassay.

Serum samples for T_3 analysis were assayed using a modified commercially available ^{125}I labeled T_3 radioimmunoassay.[q] The procedure was modified by reconstituting the antibody with a greater volume of buffer (8 vs 5 ml) and by combining ^{125}I T_3 tracer with buffer before mixing

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p Heparin vacutainer, Becton-Dickinson Division, Becton Dickinson and Co, Rutherford, NJ.
q Radioimmunoassay for histamine was performed in the laboratory of Dr. W. Hook, National Institute of Health, Bethesda, Md.
r Technicon Corp., Tarrytown, NY.

with the sample. The incubation procedure was also modified so that initially the sample stood at 37 C for 30 minutes, was chilled to 0 C for 10 to 15 minutes and then left overnight at 4 C. Samples were eluted against 0.5 ml of cold charcoal solution for 10 minutes. Following centrifugation for 10 minutes at 3200 rpm, each tube (which contained 100 ml of ^{125}I tracer solution) had 75 mg of 8-anilino-1-naphthalene sulfonic acid[s] (ANSA) added. Sensitivity of the assay at 90% of total of the standard curve was 0.28 ng/ml. Intra-assay coefficient of variability of the T_3 test with bovine serum was $\pm$ 6.5% and inter-assay coefficient of variability was $\pm$ 1.3%.

Serum T_4 was assayed using a modified commercially available[t] solid phase ^{125}I labeled T_4 radioimmunoassay. A sample of 30 µl was used to which 0.23 ng of ANSA was added, in order to increase sensitivity. Incubation took place over 2 hrs at 37 C before decanting the tubes. The sensitivity of the T_4 assay was 1.5 ng/ml at 90% of the standard curve. Intra-assay coefficient of variability was $\pm$ 4.3% and inter-assay coefficient of variation $\pm$ 5.1%. The assay had complete cross reactivity (100%) with thyroxine isomers but negligible cross reactivity to DL-Thyronine (0.04%). Reverse T_3 had 14% cross reactivity.

Cortisol was assayed using a modified commercially available[u] solid phase ^{125}I labeled cortisol radioimmunoassay (RIA). The procedure was modified for use with bovine plasma by taking larger samples volumes (20 µl), adding an additional 0.2 mg 8-anilino-1-naphthalene sulfonic acid

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s ANS, Sigma Chemical Co, St. Louis, Mo.
t T_4 solid phase radioimmunoassay, Becton-Dickinson Immunodiagnostics, Orangeburg, NY.
u Clinical Assays, Cambridge, Mass.

(ANSA) per sample tube, and incubating at 37 C for 2 hours before decanting the tubes. Specificity tests of the antiserum indicated 65.8%, 3.8% and 2.1% cross reactivity for Prednisolone, Prednisone and Corticosterone, respectively. Cross reactivity was <1% for Cortisone, Deoxycorticosterone, Dexamethasone, Progesterone and Betamethasone. Precision on replicated quality control samples indicated <10% intra and interassay coefficients of variation. Sensitivity, as calculated from the standard curve at 90% of total trace binding was 3.8 ng/ml and calculated from 0 $\pm$ 2 s.d. was <1 ng/ml. Dilution and recovery studies indicated results which supported the validity of the assays for T_3 T_4 and cortisol.[v]

Serum Specifity of Antibodies Against P. haemolytica. A single serum sample was taken from each animal during the baseline data collection period in the Pasteurella group. These serums were analyzed for cross reactivity to somatic and capsular antigens[w] of the strain of P. haemolytica used to challenge the calves. Titers to capsular antigens were determined by hemagglutination and those to somatic antigens determined by agglutination to specific rabbit antibovine globulin.

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v Radioimmunoassay for T_3 T_4 and cortisol was performed in the laboratory of Dr. R. Nachreiner, Michigan State University, East Lansing.

w Courtesy of Dr. C. Smith, Ohio Agricultural Research and Development Center, Dept. of Veterinary Science, Wooster, Ohio.

RESULTS

There was no alteration in the total white cell count (WBC) in the control group of calves during the course of the experiment. In the Pasteurella group, WBC had decreased significantly by T = 3, and was less than the control group at T = 2, T = 3 and T = 6 hrs. (Figure 2-1) The decline in WBC in the Pasteurella group was caused by a significant decrease in numbers of segmented neutrophils. In the control group of calves, segmented neutrophil numbers remained constant. (Figure 2-2) Nonsegmented neutrophil numbers were not significantly different in the control calves compared to the calves in the Pasteurella group. Both groups had significantly increased by T = 6 compared to stress but no significant differences between baseline measurements and any other measurement period were found.

There were no significant differences between groups of calves or over time for monocytes, mean $\pm$ SEM 277 $\pm$ 357 cells/mm^3), eosinophils (2 $\pm$ 1 cells/mm^3) basophils (9 $\pm$ 3 cells/mm^3) or lymphocytes (2940 $\pm$ 951 cells/mm^3). Plasma total solids (Figure 2-3), hematocrit (Figure 2-4), total erythrocyte count (Figure 2-5) and hemoglobin content (Figure 2-6) had significant and similar declines during the course of the experiment in both groups of calves. No effect of Pasteurella exposure was found. Mean corpuscular volume (mean $\pm$ SEM = 36.7 $\pm$ 2.5 μ^3), mean corpuscular hemoglobin (mean $\pm$ SEM = 13.1 $\pm$ 0.7 μg) and mean corpuscular hemoglobin concentration were unchanged during the course of the experiment but mean corpuscular hemoglobin concentration was slightly but significantly less in the Pasteurella group of calves (35.5 g/dl compared to 37.2 g/dl). The differences between the calf groups were not significant for any individual measurement period, therefore suggesting minor

Figure 2-1. Alterations in blood total white cell count in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

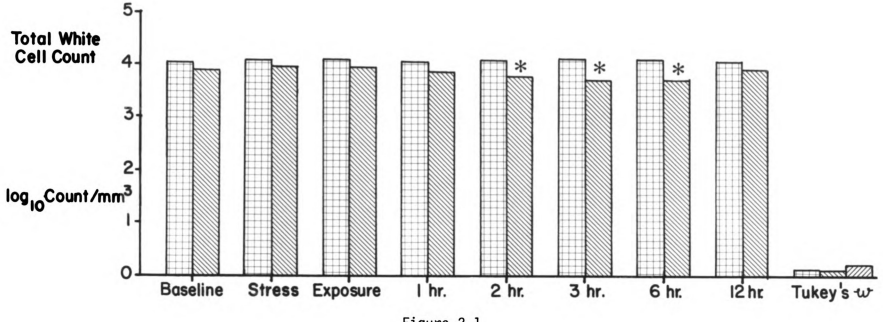
stress = immediately following chilling with cold water and spraying of the trachea with acetic acid.

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column) for the Pasteurella group (first diagnoally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.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Tukey's w
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Figure 2-1

Figure 2-2. Alterations in the blood segmented neutrophil count in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid.

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group).

1 hr = one hour after the exposure period
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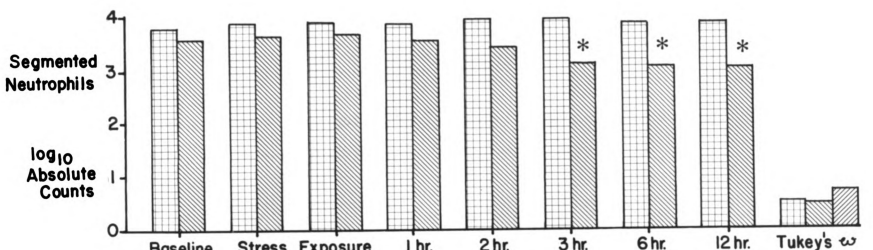
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column) for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*Denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.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4
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2
1
0
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Baseline
Stress
Exposure
1 hr.
2 hr.
3 hr.
6 hr.
12 hr.
Tukey's ω

Figure 2-2

Figure 2-3. Alterations in plasma total solids in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

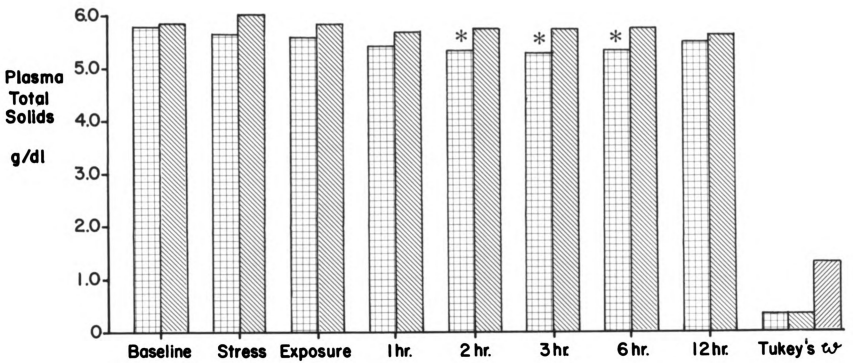
stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.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1 hr.
2 hr.
3 hr.
6 hr.
12 hr.
Tukey's w

Figure 2-3

Figure 2-4. Alterations in blood hematocrit in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

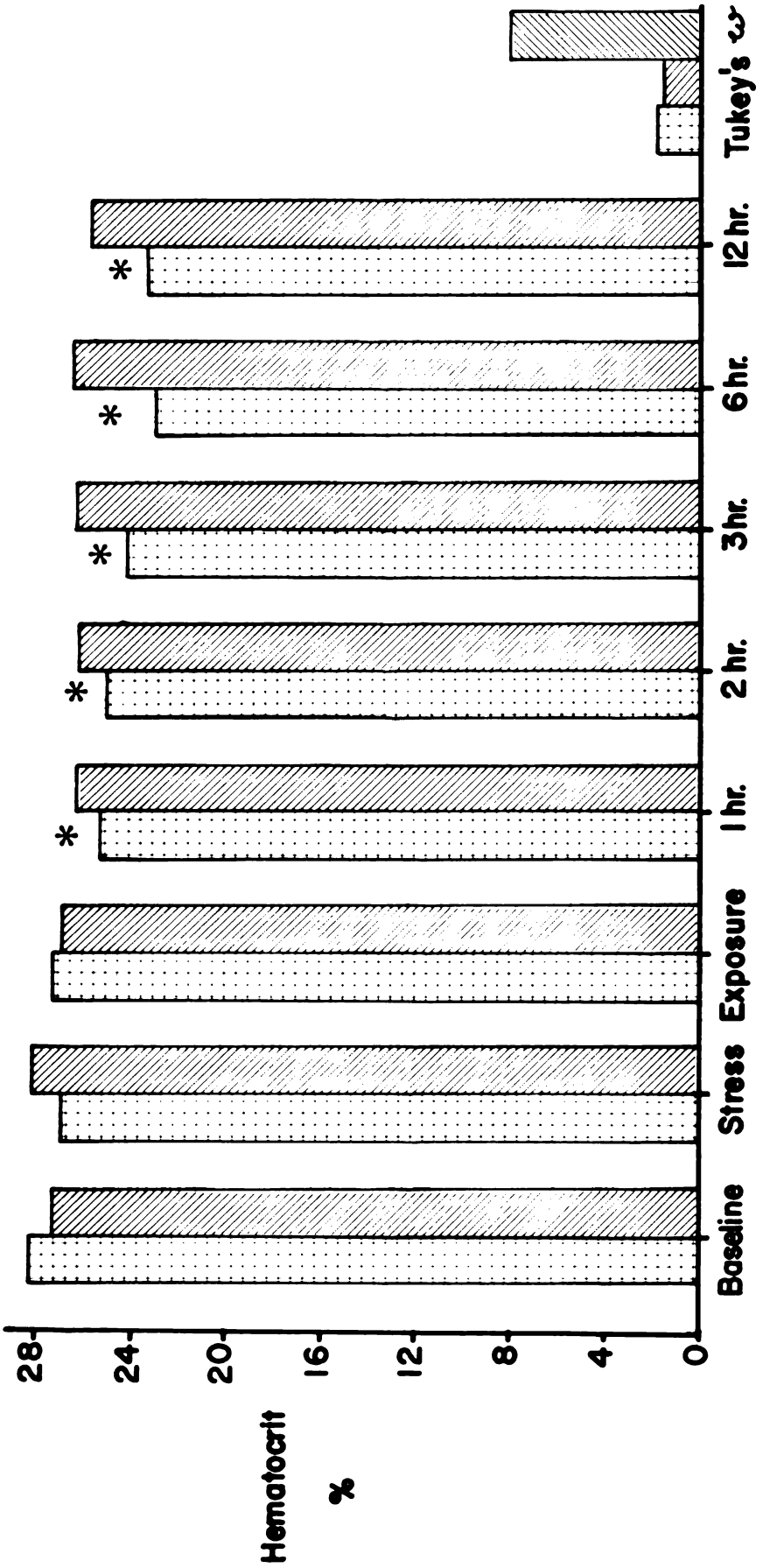
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Figure 2-4

Figure 2-5. Alterations in blood erythrocyte count in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

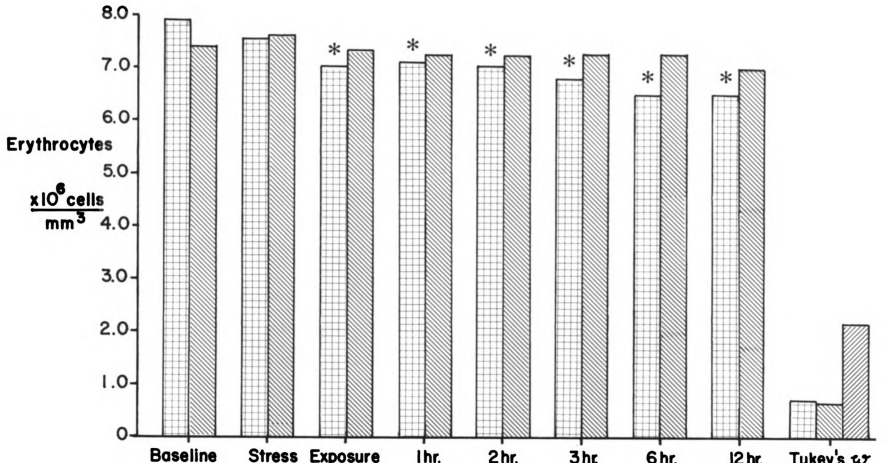
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
Erythrocytes
x10^6 cells
mm^3
*
Baseline
Stress
Exposure
1hr.
2hr.
3hr.
6hr.
12hr.
Tukey's

Figure 2-5

Figure 2-6. Alterations in blood hemoglobin content in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

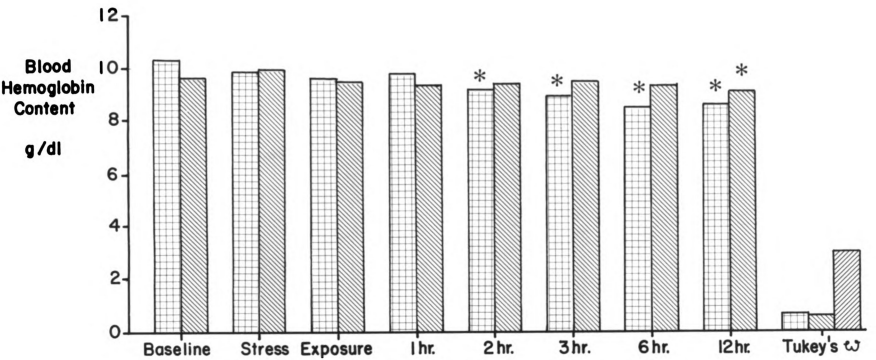
Blood Hemoglobin Content g/dl
12
10
8
6
4
2
0
*
Baseline
Stress Exposure
1hr.
2hr.
3hr.
6hr.
12hr.
Tukey's w

Figure 2-6

differences in hemoglobin concentration existed between the two groups of calves, unassociated with the development of pasteurellosis.

Serum triiodothyronine (T_3) was significantly larger in the Pasteurella calves than the control group even before exposure to Pasteurella haemolytica. (Figure 2-7) Both groups of calves had decreases in serum T_3 during the course of the experiment. No specific effect of P. haemolytica on T_3 serum concentration was detected and there was no difference between arterial and venous serum T_3 levels. Taken on an individual measurement period basis, T_3 differed significantly between control and Pasteurella calves only at the stress period being greater in the Pasteurella group.

Serum thyroxine levels (T_4) were altered similarly to T_3 over the course of the experiment (Figure 2-8) and were also greater in Pasteurella calves even during baseline measurements. As with T_3, differences in T_4 levels between groups of calves were not different for the latter half of the study.

Serum cortisol levels are illustrated in Figure 2-9. Serum cortisol levels of control and Pasteurella calves were not significantly different under baseline conditions. In the control group, stress was associated with a rise in serum cortisol levels, not significant by either LSD or Tukeys' test. Serum cortisol rose significantly with stress in the Pasteurella group. The difference in cortisol levels between calf groups at the stress measurement period was not significant. In control calves, cortisol steadily decreased from stress values over the remaining portion of the experiment. However, Pasteurella exposed calves maintained cortisol levels at or greater than stress levels for the remainder of the experiment.

Figure 2-7. Alterations in serum triiodothyronine (T_3) in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

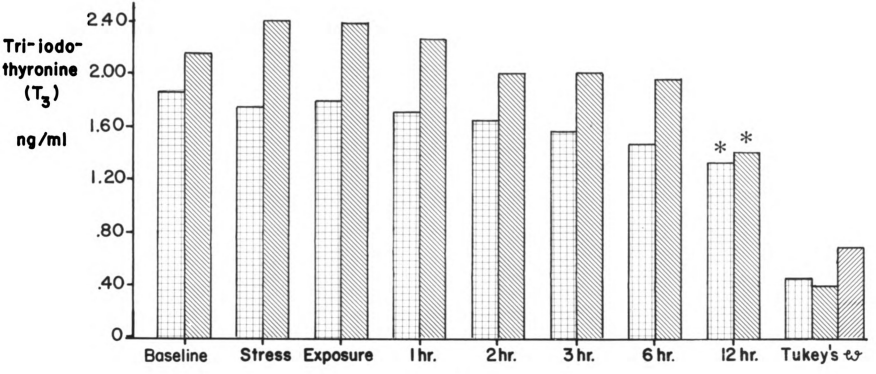
Tri-iodo-thyronine (T_3) ng/ml
2.40
2.00
1.60
1.20
.80
.40
0
*
*
Baseline
Stress Exposure
1 hr.
2 hr.
3 hr.
6 hr.
12 hr.
Tukey's w

Figure 2-7

Figure 2-8. Alterations in serum thyroxine (T_4) in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally columns). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

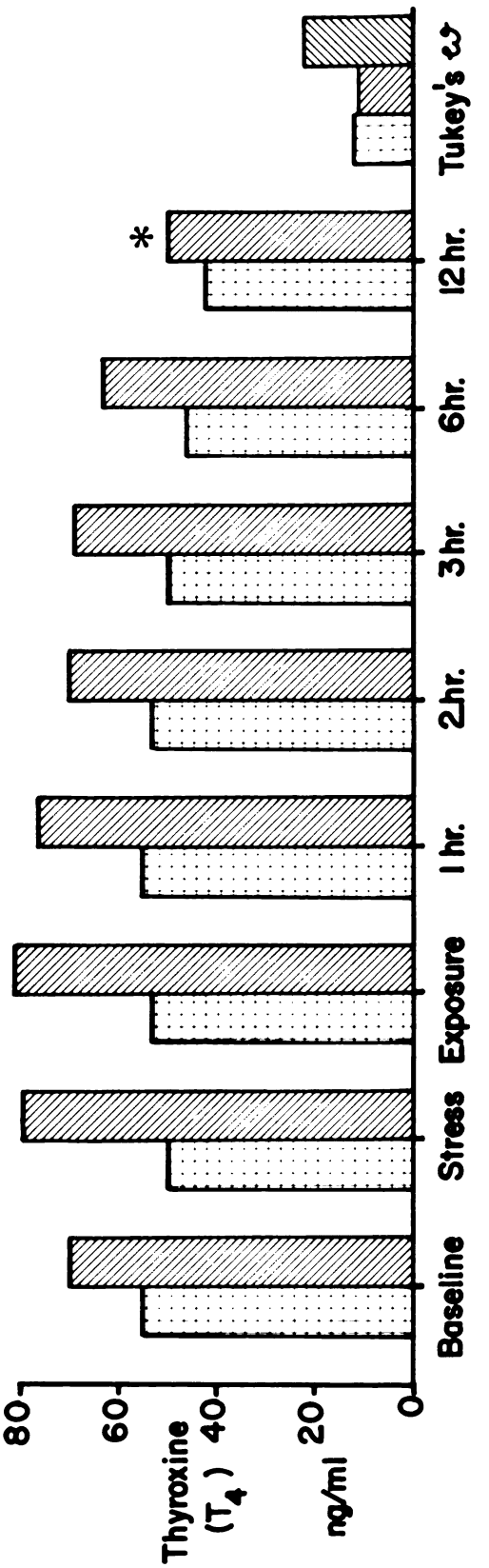
Thyroxine (T_4) ng/ml
80
60
40
20
0
Baseline
Stress
Exposure
1 hr.
2hr.
3hr.
6hr.
12hr.
Tukey's
*

Figure 2-8

Figure 2-9. Alterations in serum cortisol in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

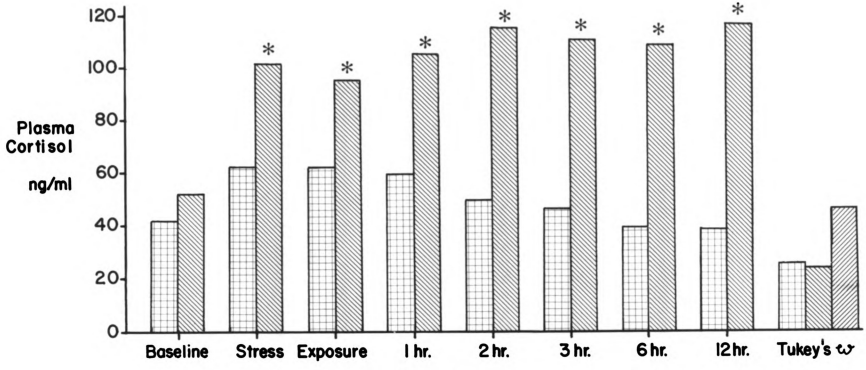
Plasma
Cortisol
ng/ml
120
100
80
60
40
20
0
*
Baseline
Stress
Exposure
1 hr.
2 hr.
3 hr.
6 hr.
12 hr.
Tukey's ω

Figure 2-9

There was a significant difference between arterial and venous plasma histamine, with venous samples having greater values (3.09 ± 0.12 ng/ml and 2.89 ± 0.11 ng/ml for venous and arterial samples respectively). There were no significant differences between groups of calves and no detected changes in histamine levels associated with P. haemolytica exposure. Both groups had small declines in histamine levels with a minimum value reached by T = 2. Plasma histamine rose after T = 3 so that peak histamine levels were present in both groups of calves by T = 12.

Plasma bradykinin levels were only determined in the Pasteurella exposed group of calves. There were no arteriovenous differences and no significant effects of exposure to P. haemolytica (mean ± SEM = 1.70 ± 0.86 ng/ml).

Serological titers to the capsular antigens of P. haemolytica were determined for the Pasteurella calves by hemagglutination test. All titers were 1/8 or less and were considered negative. Serologic titers for the somatic (O) antigens were also determined in the Pasteurella calves by agglutination to specific rabbit antibovine globulin. Low level positive titer (1:2 or less) were obtained in 3/7 calves, with remaining calves having negative titers.

DISCUSSION

During the course of the experiment, an estimated 20 to 25% of blood volume was removed for the various samples and was replaced with normal saline. Because of the removal of blood cells and dilution of plasma crystalloids with saline, we anticipated a reduction in hematocrit, total solids, erythrocyte and leukocyte counts and hemoglobin

concentration. With the exception of total white cell count, the changes in these hematologic variables were identical for both groups of calves, indicating that the changes were due to sampling and that intrapulmonary challenge with P. haemolytica does not result in erythrocyte damage or loss of plasma proteins.

The lack of a decrease in total white cell counts in control calves indicates sufficient leukocyte mobilization into the circulating pool to replace those lost by repetitive blood sampling. In contrast, calves exposed to P. haemolytica had decreased total leukocyte numbers exceeding anticipated sampling effects. Pasteurella infection might cause this change by either increased utilization of leukocytes or by failure of their replacement in the circulating pool. Our data suggests that the decline in leukocyte numbers is caused by a decrease in circulating neutrophils and from our histologic studies (see Chapter 4) indicates that these cells aggregate in very large numbers in the lungs. There was an increase in band neutrophils in the circulating blood of Pasteurella calves by T = 12 but this response was insufficient to replace granulocytes lost from the circulation.

Lymphocyte, monocyte, eosinophil and basophil numbers failed to change significantly from baseline levels in both groups of calves suggesting that these cells are less important in the initial response to P. haemolytica than are neutrophils. This suggestion is supported by histologic findings, since the former cells are not consistently featured in the inflammatory response noted in the lungs of calves exposed to P. haemolytica.[1]

The normal neonatal calf leukogram and hemogram have been well described and are reviewed in current veterinary texts of clinical

pathology.[9,10] Baseline values of the leukogram and hemogram of the calves in our study are similar to those reported in these reviews[9,10], with large numbers of circulating leukocytes, primarily neutrophils when compared to adult cattle. Routine perinatal stresses are cited as responsible for causing this leukogram.[9] These stresses are throught to release corticosteroids which, from inference to studies in older cattle, depress lymphocyte and eosinophil numbers and increase neutrophil numbers.[9,10,11] If the leukograms of neonatal calves are indeed dictated by corticosteroid levels, it is surprising that increased plasma cortisol levels induced by cold stress were not associated with changes in either the differential or total white cell count. Our data suggests that the bovine neonatal leukogram does not respond in a linear dose-response relationship to cortisol exposure. This hypothesis is supported by failure of repeated doses of ACTH to have additive effects on the leukogram of young calves.[11] Also, adult cows which were injected with corticosteroid doses ranging from 20 to 500 mg have similar alterations in their leukograms[9], despite 25 fold differences in steroid dose.

Since the alterations in serum cortisol of neonatal calves did not produce associated changes in the leukogram, the neutropenia observed in calves with Pasteurella infection is probably not due to cortisol related effects. Endotoxin, a component of gram negative bacterial cell walls, causes leukopenia, pulmonary edema, fever and accumulation of leukocytes in the lungs of calves injected with *E. coli* endotoxin.[12,13] Since each calf in our study received 2×10^9 *P. haemolytica* organisms, this represents a considerable load of endotoxin even if no further bacterial replication occurred. It is likely that endotoxin from

Pasturella haemolytica contributes to the pathogenesis of pasteurellosis by stimulating rapid migration of leukocytes into the lungs and causing vascular damage. To date the extent of that contribution is unknown.

We speculated that thyroid hormones might exert an influence on the pathogenesis of pulmonary injury in calves because hypothyroidism is associated with neonatal pulmonary disease in human infants[14] and because thyroid hormones may indirectly affect the metabolism of histamine.[15] In other species thyroid hormones increase in response to chronic rather than acute deviations in environmental temperature so it was therefore not surprising that plasma T_3 and T_4 of calves remained unchanged after cold stress.[16] Pasteurella infection had no effect on plasma T_3 and T_4. Since plasma T_3 and T_4 levels do not necessarily reflect thyroid hormone activities in peripheral tissues[17,18], the failure to detect changes in plasma T_3 and T_4 levels from normal subsequent to Pasteurella infection does not exclude their role in the pathogenesis of Pasteurella injury.

There were significant differences between the T_3 and T_4 levels of the two groups of calves unrelated to P. haemolytica exposure. These differences may relate to seasonal variations in thyroid hormone levels, since the Pasteurella group of calves was studied over the winter months, when hormone levels are greatest, and the control group of calves was studied in early spring, when thyroid hormone levels reportedly decrease below winter levels.[19] Although adaptation to cold stress is generally cited as an explanation for maximal thyroid hormone levels in winter, calves in this study were not exposed to winter temperatures. Bobeck et al (1980) described peaks in thyroid secretion of calves born in winter but never exposed to outside environmental

temperatures, indicating as in our study, that plasma thyroid hormone levels are affected by seasonal factors other than temperature.[19]

Serum T_3 and T_4 levels decreased significantly during the course of the experiment in both groups of calves. Since baseline, stress and T = 0 measurements were taken in the morning, with remaining measurement periods extending into the evening, diurnal variations in serum T_3 and T_4 may account for the declining thyroid hormone values observed over the course of the experiment. Diurnal patterns of thyroid hormone secretion have not been determined in neonatal calves, and although differences occur with the extent of diurnal rhythm between species, they are certainly present in older cattle, persons and rats.[20-22]

Interpretation of our data is further complicated by the influence of plasma dilution during sampling and by declines in serum T_3 and T_4 levels which occur from the time of birth up until approximately one week of age when values approach adult levels.[23,24]

Cold stress in calves resulted in increased serum cortisol. This was expected since a number of researchers have demonstrated the responsiveness of the young calf adrenal to various stressful stimuli.[11,25-27] The decline in cortisol levels subsequent to cold stress in control calves is probably the result of the interaction of the following factors; decline in baseline cortisol secretion rates which occur in the first few weeks of neonatal life[28,29]; dilution of plasma during blood sampling; removal of the stressful stimulus; diurnal variations in the secretion of corticosteroids.[30,31] These influences would similarly affect calves in the Pasteurella group and yet this group of calves had cortisol levels maintained at or above stress measurement levels. Acute P. haemolytica infection therefore stimulates

cortisol secretion in some manner. "Stress" through disease, resulting in elevated cortisol levels is similarly described for neonatal calves suffering from diarrhea.[29] Although the increased cortisol levels may assist indirectly in recovery from the infection by facilitation of uptake of ingested immunoglobulin[32] and in stabilization of cell membranes in response to injury[33], corticosteroid increases may cause adverse sequelae through immune suppression and loss of the ability to efficiently dispose of bacteria in the lungs. The exact role of corticosteroids in modulating immune function of calves is unclear. For example, administration of corticoids to calves is associated with lymphopenia but also with improved interferon responses to viral exposure[34], even though interferon is thought to be produced by lymphocytes. The effect of postnatal corticosteroid exposure on pulmonary growth and differentiation in calves is unknown but may be of importance. The administration of corticoids to premature human neonates aids survival by stimulating surfactant production, but postnatal administration may be detrimental by reducing pulmonary growth and by other steroid related changes in the body.[35]

In other species, infectious disease processes may involve the so-called "mediators of anaphylaxis" in pulmonary injury.[36,37] We were curious to determine whether the mediators histamine and bradykinin contributed to the pulmonary injury induced by pasteurellosis.

Pasteurellosis was not associated with an increase in plasma histamine. The interpretation of plasma histamine determinations as they relate to important physiologic events is more difficult than for bradykinin for the following reasons; plasma histamine does not represent histamine kinetics from a single body source or compartment. Most

histamine is synthesized in connective tissue mast cells and only a proportion of histamine released from this source reaches the vascular pool. Since the biological effects of extravascular histamine may be pronounced, it is unclear whether biological effects of histamine arising from mast cell degranulation is adequately reflected by changes in plasma histamine concentrations. A second source of histamine arises from the non-mast cell sources. It is produced in association principally with exocrine gland function and increased plasma levels occur as a result of cholinergic stimulation or decreased corticosteroids in plasma.[38] Alterations in this plasma pool may occur rapidly in response to cholinergic stimuli, but may not necessarily be associated with an increase in tissue histamine levels within the lung. Lastly, the biologic significance of a certain level of plasma histamine may differ, depending on the presence or absence of other mediators, autonomic influences and tissue viability. Therefore, the failure to detect significant increases in plasma histamine associated with P. haemolytica pneumonia does not exclude a possible role for pulmonary mast cells in the pathogenesis of infectious pneumonia, but does exclude the influence of circulating histamine originating from body sources outside the lungs. Assuming pulmonary mast cells do expose the lungs to high local tissue levels of histamine during pasteurellosis, it remains unlikely that administered antihistamines, which must diffuse down a concentration gradient from the vascular space into the lung tissue, can reach significant concentrations to prevent the noxious effects of histamine. Therefore, any therapeutic value to antihistamine administration in cattle affected with pasteurellosis is probably unrelated to antihistaminic drug action.

There was a significant arteriovenous difference in plasma histamine across the lung, with samples of blood from the vena cava having greater values than carotid arterial blood. This finding does not necessarily imply histamine catabolism by pulmonary tissues, since the AV difference could arise as a result of intravascular catabolism from normally present intravascular histaminase. In addition, since afferent blood to the lungs contained greater numbers of leukocytes than efferent blood, and since bovine leukocytes contain histamine which would be released into the plasma by cell lysis during processing, the arteriovenous difference may in part be due to cell sequestration within the lungs. As a corollary to this, if sequestration is an important component of maintained AV differences, then Pasteurella calves should have wide AV differences compared to control calves. Since AV differences were similar in both groups of calves, either histamine release from the lungs increased in order to compensate for leukocyte losses from the vasculature in Pasteurella calves, or alternately, leukocyte contributions to the AV histamine difference in neonatal calves are trivial using the assay method we describe.

The role of bradykinin in the genesis of pulmonary pasteurellosis was easier to identify than that of histamine since there are no stored sources of the mediator in tissues and in calves the effects of bradykinin on the lungs are restricted to the vasculature when bradykinin is given intravenously. Aerosol exposure with bradykinin has no effect on airways, gas exchange or vessels.[39] Because pasteurellosis is not associated with increased intravascular bradykinin, our data indicates that bradykinin is unimportant in the genesis of Pasteurella lesions in cattle.

We anticipated that bradykinin levels would rise as a result of pasteurellosis, because the following mechanisms of kinin activation and persistence seemed to apply to the situation in calves: endotoxin release from Pasteurella microorganisms leading to the activation of Hageman factor and the subsequent promotion of kinin formation[40-42], possible liberation of bacterial plasminogens and kallikreins by Pasteurella spp.[41]; liberation of leukocyte kallikrein caused by endotoxin[41]; loss of vascular integrity during pasteurellosis which limits bradykinin breakdown by angiotensin converting enzyme found in intact pulmonary vascular endothelium[43,44]; activation of bradykinin generation by hypercarbic acidosis[45]; impairment of pulmonary bradykininase (angiotensin converting enzyme) by hypoxemia.[46] The absence of an alteration in bradykinin levels indicates that possibly the bovine responses to endotoxin are different from those of other species and that kinin metabolism is not affected by gas exchange impairment as reported in other species. There are species differences in the response to endotoxin[41,47,48] and in preliminary investigations in this laboratory using an inspired gas mixture of 13% O_2, 13% CO_2 in calves exposed to bradykinin, we have not been able to identify any potentiating effect of hypoxia or hypercarbia (Slocombe, Robinson, Derksen and Ingersol, unpublished data) on pulmonary or vascular responses to bradykinin.

We determined serologic titers to P. haemolytica in Pasteurella challenged calves in order to determine if any failures in producing the disease were due to already present immunity. All Pasteurella challenged calves developed severe pasteurellosis, indicating that the low levels of serologic titers against P. haemolytica in these calves

was not protective. The antibodies responsible for this cross reactivity are probably the result of nonspecific reactivity arising from colostrally acquired immunoglobulins. While obviously not protective, one would hope that such nonspecific immunity would augment other defense mechanisms to facilitate recovery. There remains no compelling evidence to suggest such a function and under certain circumstances, antibody against Pasteurella spp. appears to have exacerbated pulmonary injury through immune mediated mechanisms.[49]

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CHAPTER 3

Pathogenesis of bovine pneumonia caused by Pasteurella haemolytica: Changes in pulmonary function with cold stress and during the development of Pasteurellosis

SUMMARY

Thirteen healthy neonatal Holstein calves were cold stressed by hosing with cold water for a period of 20 minutes, on two separate occasions twelve hours apart. At the end of each chilling, calves were injected with 0.5 ml of 5% acetic acid intratracheally. Measurements of the pattern of ventilation [tidal volume (V_T) respiratory frequency (f), minute ventilation ($\dot{V}_{MIN}$) and functional residual capacity (FRC)], gas exchange properties of the lungs [respiratory quotient (R_Q), alveolar ventilation ($\dot{V}_A$) oxygen uptake ($\dot{V}_{O_2}$), CO_2 production ($\dot{V}_{CO_2}$) dead space ventilation ($\dot{V}_D$), dead space/tidal volume ratio (V_D/V_T) arterial oxygen tension (P_{aO_2}), arterial CO_2 tension (P_aCO_2) and alveolar-arterial oxygen difference ($AaDO_2$)] and of the mechanical properties of the pulmonary system [dynamic compliance (C_{dyn}), pulmonary resistance (R_L) and total respiratory system resistance (R_{RS})] were taken at the times described in Chapter 2.

Calves responded to chilling by increasing $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ and $\dot{V}_A$. This was accomplished by increasing V_T with reciprocal decreases in f so that $\dot{V}_{MIN}$ remained constant. There was no change in C_{dyn}, R_L or $AaDO_2$.

Seven of these 13 calves were then inoculated intratracheally with of 2×10^9 organisms of P. haemolytica, the remaining calves serving as controls.

Within one hour of exposure, calves exposed to P. haemolytica had increased $\dot{V}_{MIN}$, f, V_D/V_T, and $\dot{V}_D$. There was a decrease in P_aO_2 associated with increased $AaDO_2$, but no change in P_aCO_2, C_{dyn} or R_L. By 3 hours post infection there were pronounced changes in P_aO_2 and $AaDO_2$, and C_{dyn} was reduced below baseline values. By 12 hours post

infection, calves infected with P. haemolytica had in addition to the above changes, increased R_L and R_{RS} and P_aCO_2.

Data from Pasteurella exposed calves indicates that gas exchange impairment and peripheral lung injury occurs very rapidly, and that increases in airway resistance only develop relatively late in the injury. The changes described probably arise from ventilation-perfusion mismatching in injured segments of lung. The alterations in breathing pattern associated with the development of gas exchange impairment are likely of reflex origin. Pasteurellosis in calves was clearly initiated from the parenchyma and small airways and was not the result of extension of lesions from conducting airways.

INTRODUCTION

In a companion article we describe the effect of "stress" and of Pasteurella haemolytica challenge on the circulating blood constituents of neonatal calves over a period of 12 hours following bacterial challenge. The purpose of this article is to describe changes in pulmonary function that occur following the same treatments. Alterations in pulmonary function caused by P. haemolytica have not been described in animals. The documentation of alterations of pulmonary function in humans affected with Pasteurellosis is scanty and in persons Pasteurellosis is a sporadic problem[1-4] with a different clinical and pathologic course from the typical pneumonic lesions which develop in cattle[5], sheep[6] and pigs.[7]

To date, only one study has reported changes in pulmonary function associated with one of the etiologic agents that contribute to infectious bovine respiratory disease.[8] Kiorpes et al (1978) described an

increase in respiratory resistance of calves infected with bovine Herpes 1 virus (IBR) and reported alterations in gas exchange consistent with obstructive respiratory disease resulting from virus induced damage to the major airways.[8]

Because P. haemolytica can induce pulmonary disease without the influence of other infective agents, and because it has a dominant role in the injury of bovine lungs even when other infectious agents are present[5], it was felt that investigation of the role of P. haemolytica in inducing alterations in bovine respiratory function was warranted.

METHODS

Male neonatal calves weighing between 40 and 46 kg were anesthetized for placement of arterial and venous catheters and for tracheostomies as described in the companion report. Following recovery from anesthesia, baseline measurements of pulmonary function were made in the sequence described below. Calves were restrained by leg hobbles and back straps so that they lay in sternal recumbency. All animals tolerated restraint well without sedation.

Mechanics of Ventilation. An esophageal balloon catheter[a] was passed via the ventral nasal meatus into the midthoracic esophagus. Catheter construction and response times have been previously described.[9] Transpulmonary pressure (P_L), the difference between pressure measured at the proximal end of the endotracheal tube and esophageal balloon pressure, was determined with a differential pressure transducer.[b] Air

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a Uc 516 latex balloon, Anode=Rubber Plating Co, Boling, Tex.
b Model PM-131, Statham Instruments Inc, Hato Rey, Puerto Rico.

flow rates were measured with a pneumotachograph[c]-transducer system[d] attached to the endotracheal tube. Frequency responses of the transpulmonary pressure and air flow catheter-transducer systems were matched to $\geq$ 12 Hz. Tidal volumes were determined by electronic integration of the flow signal.[e] Transpulmonary pressure, air flow and tidal volume were photographically recorded.[e] Dynamic compliance (C_{dyn}) and total pulmonary resistance (R_L) were calculated graphically according to the method of Amdur and Mead.[10] Respiratory rate was measured from the recording of tidal volume and minute ventilation calculated as the product of frequency and tidal volume.

Dynamic compliance and R_L were measured during spontaneous ventilation and during a period of controlled ventilation at 15 breaths/minute with a tidal volume of 750 ml.[f] The initial part of the recording made during controlled ventilation was always discarded because calves resisted forced ventilation for the first few breaths.

Total respiratory resistance was also measured by a forced oscillating technique. At end-exhalation, the pneumotachograph-transducer system was connected to a speaker in box system[g] driven by a sine wave generator[h,i], and sinusoidal flow oscillations were applied to the respiratory system. Endotracheal tube pressure vs. flow was plotted on an oscilloscope.[e] The oscillation frequency was adjusted until the

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c Fleisch No. 1, Dynasciences, Bluebell, Penn.
d Model PM-5, Statham Instruments, Inc, Hato Rey, Puerto Rico.
e Model VR-6 Recorder, Electronics for Medicine, White Plains, NY.
f Harvard Apparatus Co, Inc., Dover, Mass.
g Acoustic Research 305 cm speaker, Teledyne Acoustic Research, Norwood, Mass.
h Crown D 150 R Amplifier, Crown International Inc., Elkhart, Ind.
i 200s Function Generator, Continental Specialties Corp., New Haven, Conn.

pressure-flow loop closed, the closed loop was photographed and the resistance determined as the slope of the closed loop.

Pulmonary Gas Exchange. Expired gases were collected into a Krogh spirometer[j] for 3 minutes and simultaneously arterial blood samples were collected. Expired gas composition was determined with oxygen[k] and carbon dioxide[l] analyzers. Blood gas tensions were determined with a pH/blood gas analyzer.[m] Alveolar gas tension, dead space-tidal volume ratio (V_D/V_T), respiratory exchange ratio (R), alveolar-arterial oxygen difference ($AaDO_2$), CO_2 production ($\dot{V}_{CO_2}$), O_2 consumption ($\dot{V}_{O_2}$) alveolar ventilation ($\dot{V}_A$) and dead space ventilation ($\dot{V}_D$) were calculated using standard respiratory gas equations.[11,12] (Appendix A)

Functional Residual Capacity. Functional residual capacity (FRC) was determined by Helium equilibration. One liter of a 15.3% He-air mixture was added to the respiratory system, at the point of passive end-exhalation, using a super syringe.[n] The gas in the syringe was equilibrated with the gas in the lungs by slowly withdrawing and then reinjecting the 1 liter volume of gas in the syringe 10 times. The concentration of He was determined[o] from gas obtained from the 10th refill of the syringe. FRC was calculated as

$$FRC = (He_{initial}/He_{final})-1.$$

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j Krogh spirometer, Warren E. Collins Inc., Braintree, Mass.
k Beckman OM-14 Oxygen Analyzer, Beckman Instruments Inc., Fullerton, Calif.
l Beckman LB-2 CO_2 Analyzer, Beckman Instruments Inc., Fullerton, Calif.
m Model 1L-713 Digital pH/Blood gas analyzer, Instrumentation Laboratory, Inc., Lexington, Mass.
n Model 86303 Supersyringe, Hamilton Co., Reno, Nev.
o Collins Helium Analyzer, Warren E. Collins Inc., Braintree, Mass.

Experimental Protocol. The measurements described above were made during a baseline period, following "stress" and at 0, 1, 2, 3, 6 and 12 hours subsequent to challenge either with sterile saline or with P. haemolytica suspended in saline. The methods of "stress" and of challenge at 0 hour are described in a companion paper in detail. Briefly, calves were stressed by exposure to cold water hosing twice, for two periods of 20 minutes, 12 hours apart. At the end of each period of chilling calves received 0.5 ml of 5% acetic acid intratracheally. Calves were either challenged with saline containing P. haemolytica or with sterile saline, by intratracheal injection.

Recordings of P_L, air flow and tidal volume were collected at the start of each measurement period, during spontaneous ventilation. Expired and blood gas samples were collected during this time for evaluation of gas exchange. Air flow, tidal volume and P_L were recorded again during controlled ventilation and finally FRC was determined.

Statistical Analysis. Data were analyzed using factorial analysis of variance. If F values were significant at $p < 0.05$, means from each measurement period were compared using Tukey's ω and LSD tests.[13]

RESULTS

Tidal volume (V_T) was not altered by Pasteurella exposure. Tidal volume was increased in both groups of calves during the stress measurement period, remained increased for the T=0 measurement, but had returned to baseline by T=1. (Figure 3-1). Respiratory rate (f) was decreased during the stress time period but had returned toward baseline levels by T=0. Respiratory rate remained at baseline levels for the

Figure 3-1. Alterations in tidal volume in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

control = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

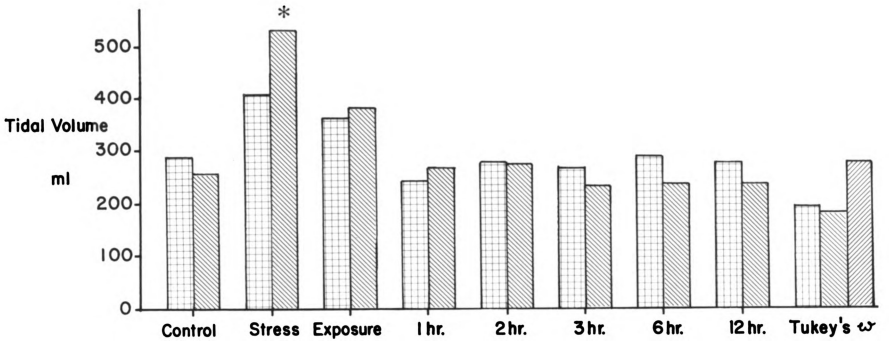
Tidal Volume
ml
500
400
300
200
100
0
*
Control
Stress
Exposure
1 hr.
2 hr.
3 hr.
6 hr.
12 hr.
Tukey's w

Figure 3-1

Figure 3-2. Alterations in respiratory rate in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

control = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
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6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

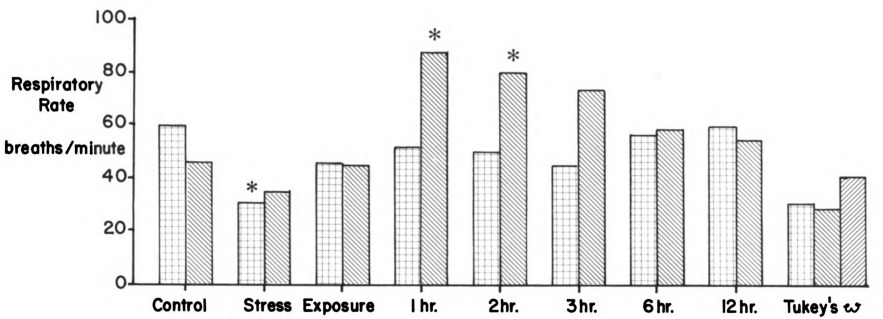
Respiratory Rate
breaths/minute
100
80
60
40
20
0
*
*
*
Control
Stress
Exposure
1 hr.
2hr.
3hr.
6hr.
12hr.
Tukey's w

Figure 3-2

remainder of the experiment in the control group but was significantly increased at T=1, T=2 and T=3 measurement periods in the Pasteurella exposed calves compared to the stress measurement period. (Figure 3-2). As a result of these changes in f and V_T, minute ventilation was unaltered during stress in either group of calves, and remained at baseline values for the entire experiment in the control group. In contrast, the Pasteurella group increased minute ventilation significantly at T=1 compared to the control group. Although differences in minute ventilation from baseline remained large at T=2 and T=3, the increase above baseline was not significant. (Figure 3-3).

Alveolar-arterial oxygen difference ($AaDO_2$) remained unchanged in the control group of calves. In the Pasteurella exposed group a trend toward increasing $AaDO_2$ was noted by T=2 (significantly different from baseline on LSD but not Tukey's ω test) and by T=3, $AaDO_2$ was significantly greater than baseline. Differences from baseline were significant at T=6 but by T=12, $AaDO_2$ had decreased so that it was no longer significantly different from baseline values, yet remained increased enough above baseline that it was not significantly different from T=6. (Figure 3-4).

Changes in PaO_2 reflected the alterations in $AaDO_2$. There were no changes in the control calves and in the Pasteurella group of calves the decrease in PaO_2 was significantly different from baseline by T=1. Further declines in PaO_2 over the remainder of the experiment were small so that measurements after T=1 were not significantly different from T=1, but remained different from baseline. (Figure 3-5).

Arterial CO_2 tension remained unchanged in the control group over the course of the experiment. In the Pasteurella group, $PaCO_2$ remained

Figure 3-3. Alterations in minute ventilation in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

control = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between groups for the same measurement period at the 0.05 level.

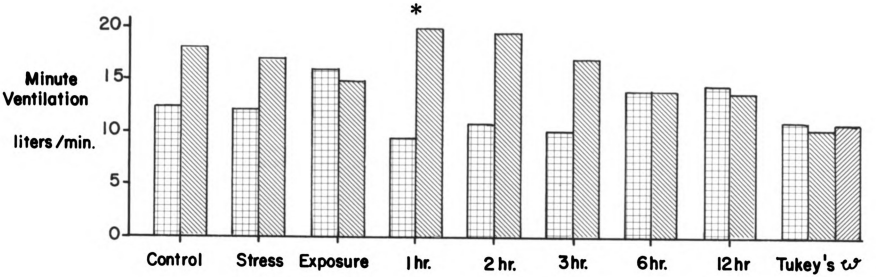
Minute Ventilation
liters/min.
20
15
10
5
0
*
Control
Stress
Exposure
1 hr.
2 hr.
3hr.
6hr.
12hr
Tukey's w

Figure 3-3

Figure 3-4. Alterations in alveolar-arterial oxygen difference in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

control = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

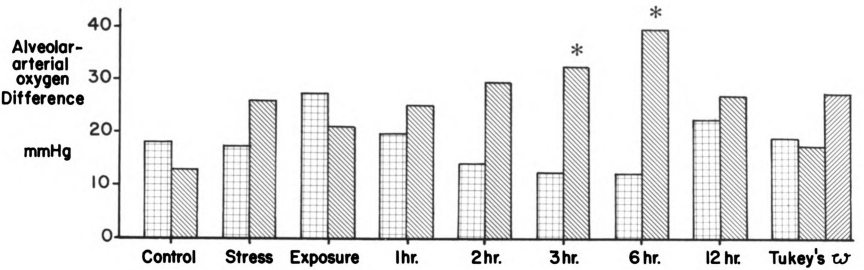
Alveolar-arterial oxygen Difference
mmHg
40
30
20
10
0
*
*
Control
Stress
Exposure
1hr.
2hr.
3hr.
6hr.
12 hr.
Tukey's w

Figure 3-4

Figure 3-5. Alterations in arterial oxygen tension in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

control = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

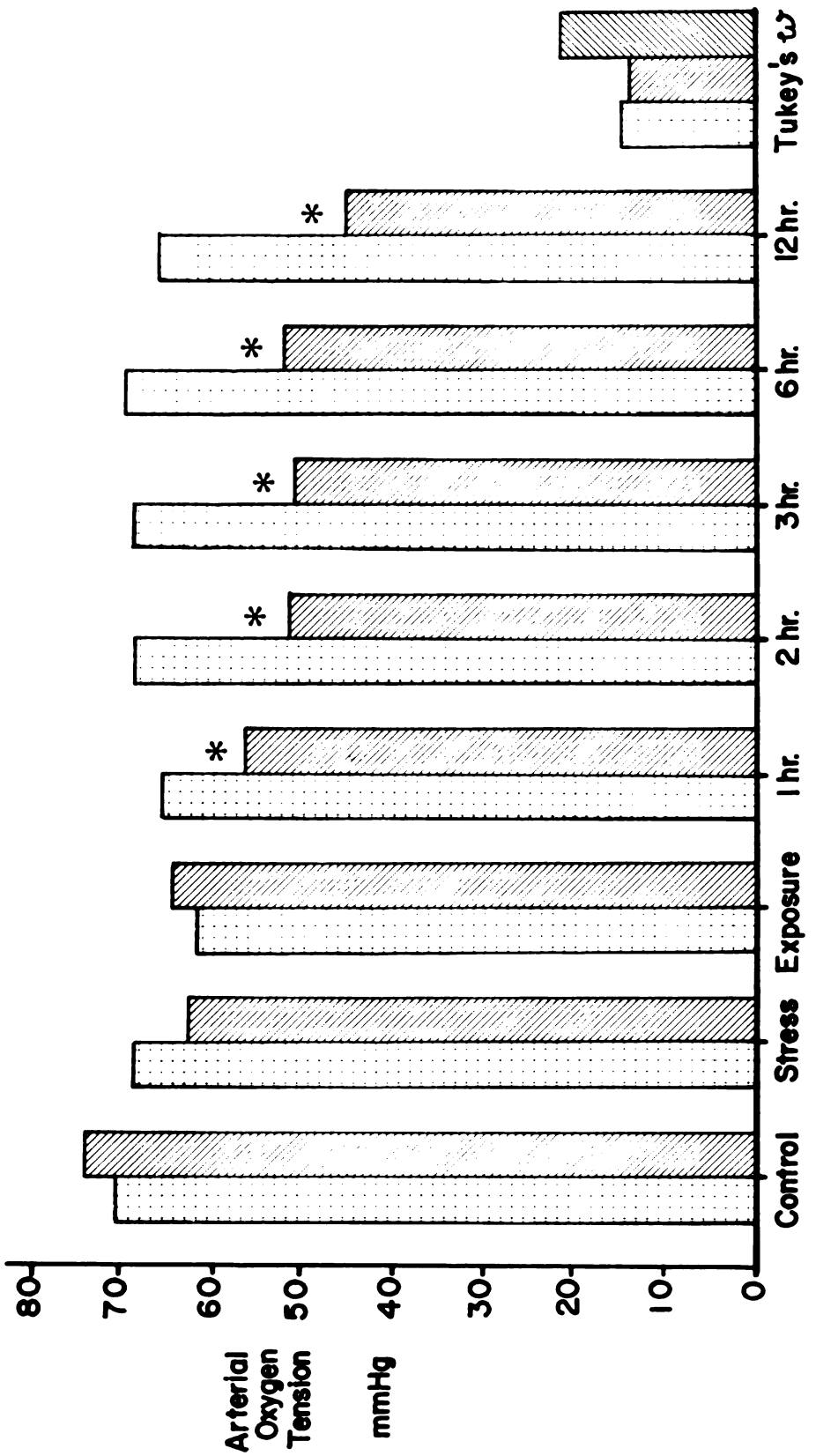
Arterial Oxygen Tension mmHg
80
70
60
50
40
30
20
10
0
Control
Stress
Exposure
1 hr.
2 hr.
3 hr.
6 hr.
12 hr.
Tukey's ω
*
*
*
*
*

Figure 3-5

Figure 3-6. Alterations in arterial carbon dioxide tension in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

control = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

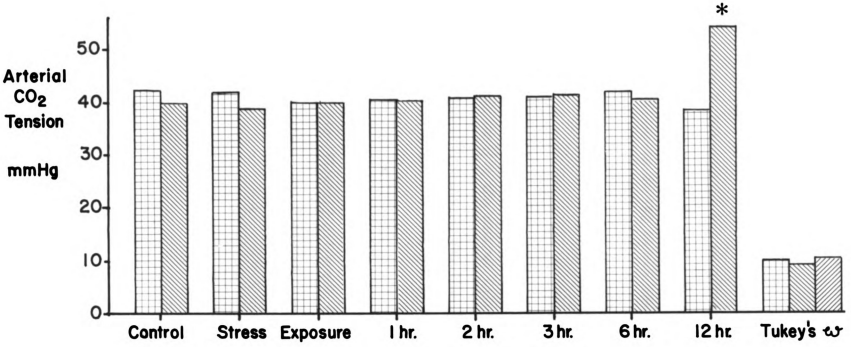
Arterial
CO_2
Tension
mmHg
50
40
30
20
10
0
*
Control
Stress
Exposure
1 hr.
2 hr.
3 hr.
6 hr.
12 hr.
Tukey's

Figure 3-6

unchanged until T=12 when $PaCO_2$ increased 14 mmHg above baseline levels. (Figure 3-6).

Production of CO_2 changed by similar amounts in both group of calves, increasing significantly during stress and T=0 measurement periods. (Figure 3-7). Exposure to P. haemolytica had no effect on $\dot{V}_{CO_2}$. Utilization of O_2 had similar changes, with $\dot{V}_{O_2}$ only increasing above baseline levels in both groups of calves during stress and T=0 measurement periods. (Figure 3-7).

Alveolar ventilation increased in both groups of calves at stress and T=0, returning to baseline levels at T=1. There was no effect of P. haemolytica exposure. (Figure 3-8).

Dead space ventilation remained constant in the control calves and was unaffected by stress. In the Pasteurella group $\dot{V}_D$ was significantly increased above baseline values at T=1, T=2 and T=3. At T=6 $\dot{V}_D$ had returned to baseline levels but during T=12, $\dot{V}_D$ was marginally increased above baseline levels (significant on LSD test but not Tukey's test). Dead space ventilation at T=12 was not significantly different from T=3 or T=6 measurement periods using Tukey's test, but differed from T=3 using the LSD test. (Figure 3-9).

The dead space/tidal volume ratio decreased significantly in both groups of calves during the stress measurement period and returned to baseline levels at T=0. In the control calves, V_D/V_T did not change from baseline levels during the remainder of the experiment. The dead space tidal volume ratio in the Pasteurella group at T=2, T=3 and T=12 hours was greater than baseline levels and was significantly larger than V_D/V_T of control calves compared at the same measurement periods. (Figure 3-10).

Figure 3-7. The effects of cold stress and intratracheal exposure to saline or P. haemolytica infected saline on CO_2 production (VCO_2) and oxygen uptake (VO_2). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

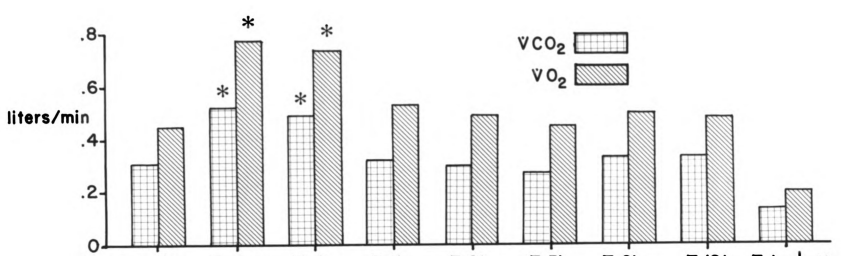
.8
.6
.4
.2
0
liters/min
$\dot{V}CO_2$
$\dot{V}O_2$
*
Baseline
Stress
T=0
T=1hr.
T=2hr.
T=3hr.
T=6hr.
T=12hr.
Tukey's ω

Figure 3-7

Figure 3-8. The effects of cold stress and intratracheal exposure to saline or P. haemolytica infected saline on alveolar ventilation. The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison of means between measurement periods is shown.

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

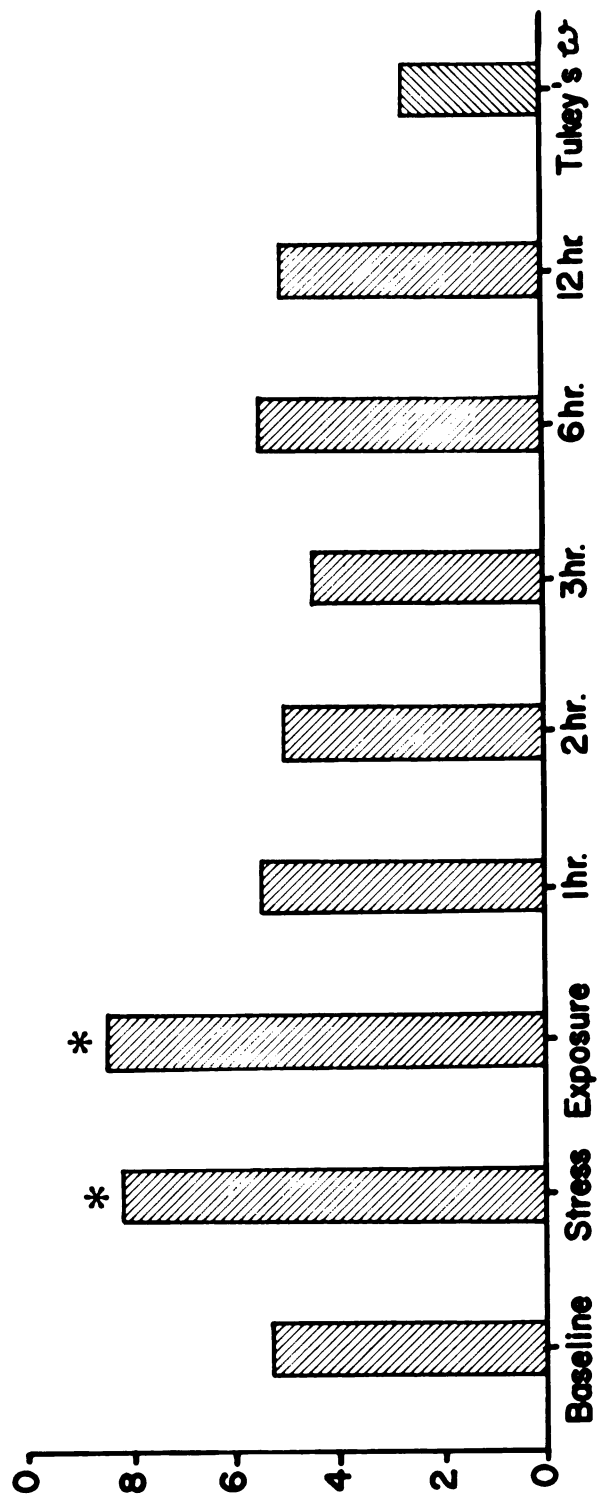
Alveolar Ventilation liters/min
10
8
6
4
2
0
*
*
Baseline
Stress Exposure
1hr.
2hr.
3hr.
6hr.
12hr
Tukey's

Figure 3-8

Figure 3-9. Alterations in dead space ventilation in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

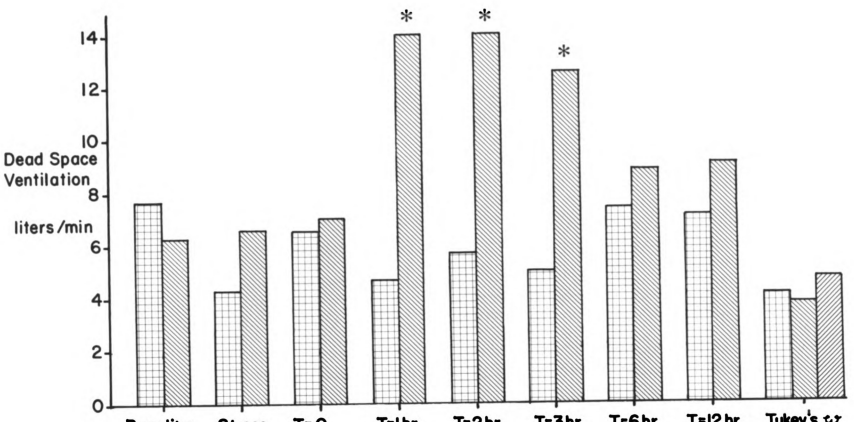
Dead Space Ventilation
liters/min
0
2
4
6
8
10
12
14
*
*
*
Baseline
Stress
T=0
T=1hr.
T=2hr.
T=3hr.
T=6hr.
T=12hr.
Tukey's w

Figure 3-9

Figure 3-10. Alterations in dead space/tidal volume ratio in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

control = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

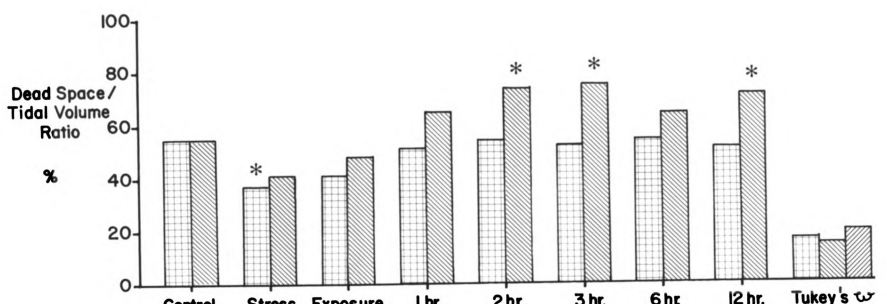
Dead Space/
Tidal Volume
Ratio
%
100
80
60
40
20
0
*
*
*
*
Control
Stress
Exposure
1 hr.
2 hr.
3 hr.
6 hr.
12 hr.
Tukey's

Figure 3-10

Respiratory quotient remained unchanged in the control group of calves, and in the Pasteurella group all measurements did not differ significantly from baseline. However, in the latter group R was increased at the T=6 measurement period when compared to the three measurement periods preceeding it. (Figure 3-11).

Functional residual capacity remained unchanged for the entire experiment in both groups of calves (mean $\pm$ SEM of all observations 2.21 $\pm$ 0.65 liters). Rectal temperature was also unchanged during the course of the experiments (mean $\pm$ SEM of all observations = 38.9 $\pm$ 1.7 C).

Dynamic compliance under spontaneous ventilation was very variable with no significant effects of Pasteurella exposure or stress detected (mean $\pm$ SEM of all observations 111.4 $\pm$ 88.2 ml/cm of H_2O). There was no difference in C_{dyn} measured during controlled ventilation for any measurement period in the control group of calves. Although C_{dyn} had decreased to less than half of baseline values in the Pasteurella group by T=12, these differences were not significant. However, C_{dyn} at T=3 was significantly less than at stress and T=1 measurement periods by LSD test but not Tukey's test, and C_{dyn} at T=6 and T=12 were significantly less in the Pasteurella group compared to the control calves. (Figure 3-12).

Total pulmonary resistance determined during spontaneous ventilation was very variable and we were unable to detect any changes associated with stress or Pasteurella infection (mean $\pm$ SEM of all observations 7.04 $\pm$ 7.06 cm of H_2O/liter/sec). During controlled conditions, R_L was significantly greater at T=12 than at baseline and T=2 measurement periods. There were no significant differences between groups of calves except at the T=1 measurement period where R_L in the control group of

Figure 3-11. Alterations in respiratory exchange ratio in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

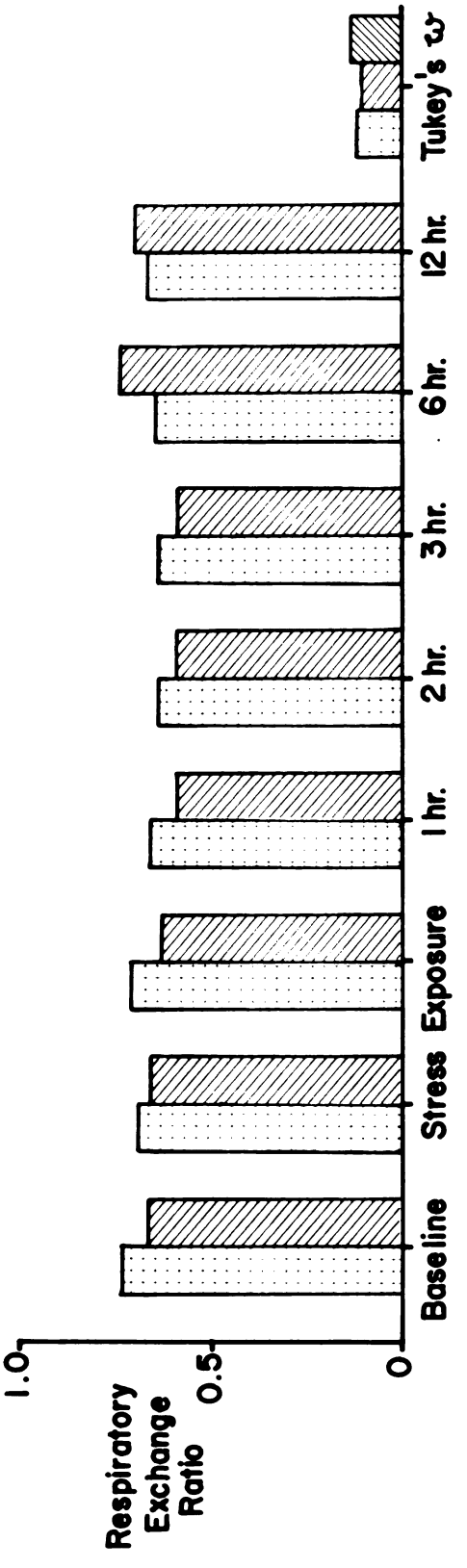
Respiratory Exchange Ratio
1.0
0.5
0
Baseline
Stress Exposure
1 hr.
2 hr.
3 hr.
6 hr.
12 hr.
Tukey's ω

Figure 3-11

Figure 3-12. Alterations in dynamic compliance in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between groups for the same measurement period at the 0.05 level.

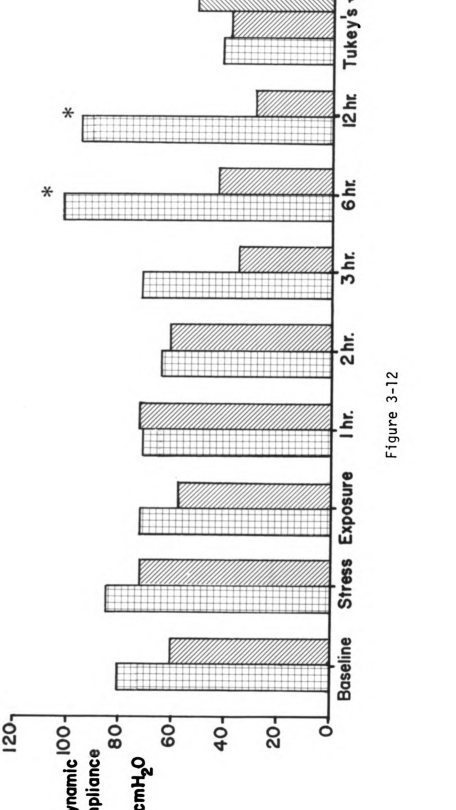
Dynamic Compliance
ml/cmH2O
120
100
80
60
40
20
0
Baseline
Stress Exposure
1 hr.
2 hr.
3 hr.
6 hr.
12 hr.
Tukey's
*
*

Figure 3-12

Figure 3-13. Alterations in total pulmonary resistance in two groups of calves; cold stress alone (square hatched columns) and cold stress combined with P. haemolytica exposure (diagonally hatched columns). The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison between measurement periods is illustrated in order from left to right, for the cold stress group (square hatched column), for the Pasteurella group (first diagonally shaded column) and for comparison between treatment groups (second diagonally shaded column)

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

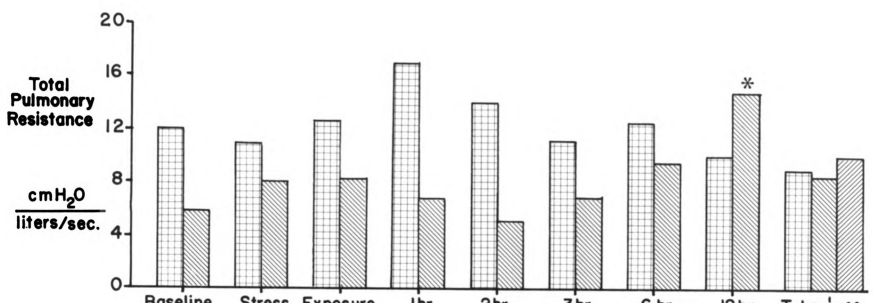
Total Pulmonary Resistance
cmH2O
liters/sec.
20
16
12
8
4
0
*
Baseline
Stress
Exposure
1hr.
2hr.
3hr.
6 hr.
12hr.
Tukey's

Figure 3-13

calves was greater than in the Pasteurella group. (Figure 3-13). However, if the total numbers of observations of R_L in each group of calves were compared, then R_L was significantly greater in the control group (12.5 $\pm$ 0.8 compared to 8.1 $\pm$ 0.8 cm of H_2O/liter/sec). We do not believe this relates to a significant difference in airway caliber between groups of calves, since three calves in the control group were fitted with endotracheal tubes of a smaller size.

Forced oscillating resistance measurements were only taken in 3/6 control calves, so that statistical comparisons between the control and Pasteurella calves were not possible using multifactorial analysis due to the wide disparity in numbers of observations between each group. Respiratory system resistance determined by forced oscillation remained unchanged in the control group of calves (mean $\pm$ SEM of control group = 9.0 $\pm$ 7.2 cm of H_2O/liter/sec) but in the Pasteurella group resistance had increased four fold by T=6 and was significantly different at T=12 hours from all preceeding measurements with the exception of T=6. (Figure 3-14).

Figure 3-14. Effects of cold stress and following exposure to P. haemolytica on forced oscillating resistance in calves. The measurement periods are:

baseline = initial baseline measurements

stress = immediately following chilling with cold water and spraying of the trachea with acetic acid

exposure = immediately after intratracheal inoculation with sterile saline (cold stress group) or P. haemolytica suspended in saline (Pasteurella group)

1 hr = one hour after the exposure period
2 hr = two hours after the exposure period
3 hr = three hours after the exposure period
6 hr = six hours after the exposure period
12 hr = twelve hours after the exposure period

Tukeys ω statistic for comparison of means between measurement periods is shown.

*denotes significant differences between measurement periods and base-line for a treatment group at the 0.05 level.

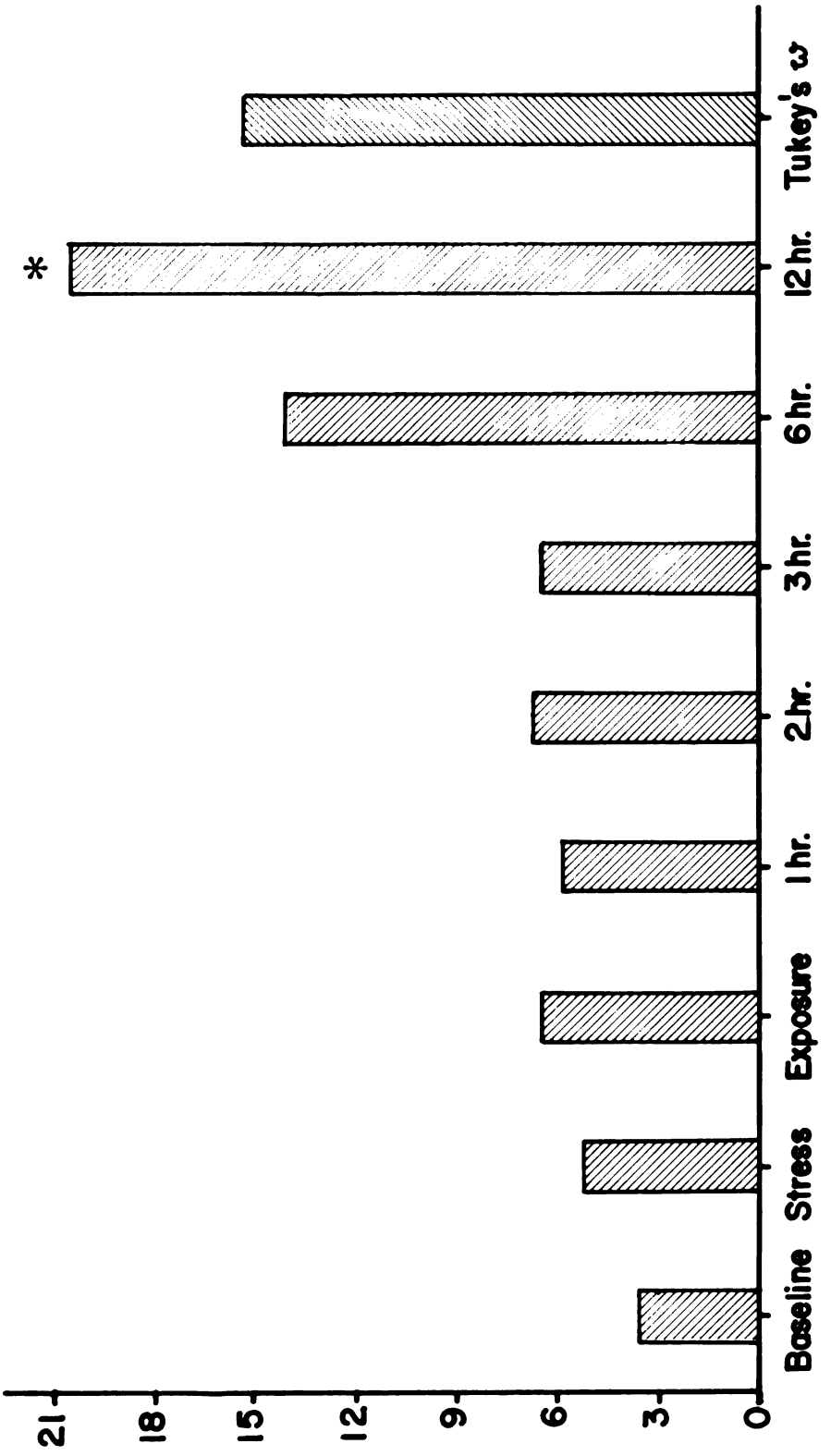
Forced Oscillatory Resistance
cmH2O
liters/sec
21
18
15
12
9
6
3
0
Baseline
Stress
Exposure
1 hr.
2hr.
3hr.
6hr.
12hr.
Tukey's ω
*

Figure 3-14

DISCUSSION

Although cold stress alone is thought to predispose to respiratory disease in cattle, clinical and epidemiologic studies suggest that chilling associated with high humidity or wetting facilitates the development and severity of the condition.[14-20] The mechanism for this predisposition is uncertain in calves, but may involve alterations in respiratory epithelial viability[21], in bacterial survival[22] and in immune function.[14] In a companion article we demonstrated that cold water chilling of neonatal calves leads to increased plasma cortisol levels and suggested that this may result in immunosuppression. Kelley (1980) indicated that cold stress may also be immunosupressive[14], but the mechanisms responsible for immunosuppression remain unclear. In this article we explored another possible avenue in which the lungs ability to respond to Pasteurella exposure may be compromised as a result of cold stress, namely pulmonary function.

Cold stress in calves induced by chilling with cold water results in immediate changes in the clinical appearance of calves so exposed. The animals become subdued, develop a "tucked up" posture and shivering begins almost immediately. These responses were effective in maintaining body temperature as measured by rectal thermometer, probably by a combination of increased body metabolism through shivering and by peripheral vasoconstriction insulating the body core from the skin, the skin surface becoming cold soon after cold water spraying began. Increased metabolism, due in part to shivering, resulted in increased $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$. In order to meet these metabolic demands without changes Pa_{O_2} or Pa_{CO_2} calves increased $\dot{V}_A$ while maintaining minute ventilation

constant. This was accomplished by increasing the depth of respiration with reciprocal decreases in respiratory frequency. As a result V_D/V_T decreased. Although such responses may serve to meet metabolic demands, the alteration in breathing pattern may serve to deposit infective aerosols further into the lungs and predispose to pneumonia. Chilling did not alter the mechanical properties of the lungs, (C_{dyn} and R_L) nor did it alter gas exchange as measured by $AaDO_2$, PaO_2 and $PaCO_2$.

It would seem probable that chilling of calves with cold water provides a greater stress than does exposure to cold environmental temperatures.[16-20] If this is so, our data suggests compromise of pulmonary defense mechanisms may be greater in the former circumstance because of large increases in circulating cortisol levels and increased exposure of gas exchange surface for a given minute ventilation.

What are the first changes in lung function caused by P. haemolytica? There were no instantaneous effects of exposure to P. haemolytica but by 3 hours post exposure PaO_2 had decreased. Because $PaCO_2$ was constant, hypoxemia was not due to alveolar hypoventilation but was due to either ventilation-perfusion mismatching or diffusion impairment as reflected by an increased $AaDO_2$. Increases in respiratory frequency combined with a tidal volume maintained at baseline levels resulted in increased minute ventilation compared to baseline at this time. The increased minute ventilation was not effective in increasing alveolar ventilation ($\dot{V}_A$ and $PaCO_2$ remained unchanged) indicating that the increased ventilation was dead space ventilation (increased V_D and V_D/V_T were observed). To determine whether this increase in dead space ventilation reflected a shift in gas distribution in the lungs or whether it was merely the result of more rapid

respiratory frequency, we calculated the dead space volume (V_D) where $V_D = \dot{V}_D/f$ at baseline and at T=1, T=2 and T=3. The volume of V_D was greater at T=1, T=2 and T=3 than at baseline by approximately 50%, indicating that alterations in $\dot{V}_D$ are not fully explained by changes in respiratory frequency alone and are due to increases in dead space volume. Increased V_D/V_T in the early stages of pasteurellosis was accompanied by increased $AaDO_2$ and indicates the development of ventilation-perfusion ($\dot{V}/\dot{Q}$) inequalities within a few hours of P. haemolytica exposure.

Two mechanisms may serve to promote the rapid and extensive development of $\dot{V}/\dot{Q}$ inequalities. Firstly, the destruction of pulmonary tissues associated with bovine pneumonic pasteurellosis involves principally the cranial lobes. As a result, anatomic dead space is probably increased as more of the inspired air is directed to caudal lobes away from poorly ventilated diseased cranial lobes.

Secondly, gas exchange impairment may be compounded by the persistence of perfusion to poorly ventilated, pneumonic regions of lung which were damaged by P. haemolytica. It would appear that, at least in dogs, bacterial products disturb local mechanisms of $\dot{V}/\dot{Q}$ matching, perhaps by impairing hypoxic vasoconstrictor responses.[23] Similar mechanisms of bacterial induced $\dot{V}/\dot{Q}$ mismatching may be present in calves. Evidence from histopathologic studies of P. haemolytica infected calves (see Chapter 4) indicates extensive perfusion of pneumonic portions of lung.

From previous studies in neonatal calves, where $AaDO_2$ and PaO_2 were determined while calves were anesthetized, we suspected that the low PaO_2 and relatively large AaO_2 were due to the combined effects of

anesthesia and thoracic restriction of motion by placement in sternal recumbency.[24] Data from this study, in which animals remained awake but were similarly positioned, suggests that anesthesia was not a factor, since $AaDO_2$ and PaO_2 are similar to values we previously described for neonatal calves.[24] The differences in $AaDO_2$ and PaO_2 between these studies and those of older calves and adult cattle[25-28] may therefore relate to age differences or differences in body position during sampling, rather than the effects of anesthesia as we had originally supposed.

Increases in respiratory rate develop at the same time that alterations in gas exchange occur. Although the decrease in PaO_2 might stimulate respiration at this time, it is unlikely, because hypoxemia is a weak stimulus for respiration in calves.[28] In healthy persons, PaO_2 usually only drives respiration at or below a carotid body PaO_2 of 50 mmHg[29], a considerably more hypoxemic condition than occurs in Pasteurellosis at T=1, and the hypoxic ventilatory drive of calves is thought to be even less sensitive than that of adult persons.[28] Since there was no change in $PaCO_2$, the most likely cause for increasing the respiratory rate is that of receptor stimulation within the lungs.

The two types of receptors most likely to be involved in altering the respiratory rate in response to pulmonary injury are irritant receptors and J receptors.[30,31] Irritant receptor stimulation usually leads to reflex brochoconstriction[30,31] and was not observed in calves at a time when respiratory rates were elevated (as indicated by no change in R_L at this time).

The function of J receptors is only partially understood, but one stimulus for receptor activation appears to be pulmonary edema.[30-32]

The severe pulmonary vascular damage associated with Pasteurella haemolytica pneumonia may lead to J receptor stimulation and initiate tachypnea. Stimulation of pulmonary receptors with vagal afferents is the cause of tachypnea associated with ovalbumin[33] and 3 methylindole[34] induced pulmonary injury of horses and with tachypnea associated vagal afferent activity of dogs after Ascaris suum antigen challenge.[35]

From the above findings by ourselves and others[33-35], it therefore appears that tachypnea observed in a variety of pulmonary injuries in several species occurs unrelated to gas exchange impairment or bronchoconstriction. We believe that a common mechanism is responsible for tachypnea under these circumstances and that it is probably due to pulmonary receptor stimulation.

The maintenance of C_{dyn} and R_L at baseline levels during the development of gas exchange impairment and alternations in respiratory rate suggests that the initial lesions of P. haemolytica pneumonia occur in peripheral lung areas rather than as an extension from central airway lesions. Studies of clearance mechanisms of P. haemolytica from calf lungs after intratracheal inoculation also indicate that lesions associated with bacterial retention occur chiefly in the pulmonary parenchyma.[36] It remains unclear whether initial lesions of naturally occurring P. haemolytica infections develop in the lung parenchyma since experimentally induced disease may preferentially deposit bacteria in lung parenchyma and therefore by association, cause initial lesions in parenchymal tissues.

By 12 hours post infection, calves had developed profound hypoxaemia and alveolar hypoventilation but $AaDO_2$ had improved compared to 6 hours

post inoculation. Such a change in $AaDO_2$ can be explained if PAO_2 declined from 6 to 12 hours post inoculation, since the effects of $\dot{V}/\dot{Q}$ inequalities become less as PAO_2 approaches the PO_2 of venous blood. Since there was no change in $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$ at 12 hours post infection compared to baseline values, the increase in $PaCO_2$ was the result of decreased $\dot{V}_A$. Because minute ventilation was unaltered from baseline, the decrease in $\dot{V}_A$ was due to increased V_D/V_T, and probably reflects respiratory failure resulting from increased work of breathing due to changes in the mechanical properties of the lungs.

By 12 hours post infection, gas exchange impairment and hypoxemia were accompanied by a decrease in C_{dyn} and an increase in R_L. Increases in R_L may result from central and/or peripheral airway obstruction through either bronchoconstriction and/or physical obstruction of airways with cellular debris, mucus and exudates. Because peripheral airways comprise a small proportion of respiratory resistance, extensive peripheral airway obstruction is necessary to cause measurable increases in R_L. From our histologic studies, it would appear that increases in R_L are probably caused by extensive peripheral airway obstruction as a result of physical obstruction with purulent exudates and edema fluid.

Decreases in C_{dyn} may occur as a result of increases in FRC, loss of parenchymal elasticity or peripheral time constant inequalities associated with small airway obstruction. Such processes are likely to exist together in calf lungs. Because bovine lungs have no collateral ventilatory pathways, peripheral airway obstruction in calves should always result in the development of peripheral time constant inequalities between pulmonary segments, atelectasis of segments with completely

obstructed airways, and hyperinflation of normal segments of lung given that FRC is unchanged.

Pasteurella infection was not associated with an alteration in FRC. Because FRC was determined by Helium dilution, it reflects the volume of gas in the thorax in communication with the endotracheal tube. Histopathologic studies on the calves infected with P. haemolytica (see companion paper) indicate that extensive areas of atelectasis, exudative pneumonia and obstructive supporative bronchiolitis had occurred by twelve hours post infection. In order for FRC to remain constant despite the development of these lesions, hyperinflation of the remaining portions of lung in communication with the mouth needed to occur. In so doing, the hyperinflation results in decreases in C_{dyn} below baseline values because tidal breathing must occur at a flattened portion of the pulmonary pressure-volume curve where lung compliance is low. Hyperinflation of healthy lung tissue probably contributed to the decrease in C_{dyn} associated with Pasteurellosis.

We describe decreases in C_{dyn} and gas exchange impairment of calves at 3 hours postexposure to Pasteurella, prior to any change in R_L, clearly showing that peripheral airways and pumonary parenchyma are involved in Pasteurellosis well before any possible involvement of central air ways. This situation obviously differs from that caused by IBR virus in calves, where increases in resistance and hypoventilation occur as a result of central airway disease, and are unassociated with gas exchange impairment and alteration in C_{dyn}.[8]

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CHAPTER 4

Pathogenesis of bovine pneumonia caused by Pasteurella haemolytica: gross and microscopic lesions

SUMMARY

Routine gross and light microscopic studies were performed on two groups of neonatal Holstein calves. The first group of 6 animals served as controls. They were subjected to cold stress and subsequent recording of hematologic and physiologic variables for 12 hrs following injection of saline into the trachea, as described in companion chapters. At the end of this period, animals were euthanatized and a gross and microscopic evaluation of each animal was performed, with particular attention being paid to the respiratory tract. Acetic acid spraying of the trachea, administered during the cold stress periods, caused severe focal necrotizing lesions in the trachea. All calves had inhaled necrotic debris, neutrophilic exudate and sloughed portions of large airway mucosa. The majority of the lungs were normal but a few lobules had partially collapsed and had variable amounts of edema, hemorrhage and neutrophilc exudates present in their lumens.

The second group of 9 calves had been exposed to P. haemolytica by intratracheal inoculation, and had died or been euthanatized at various time intervals following challenge. Calves which survived 18 hrs or longer had extensive fibrinous pneumonia with necrotic foci and an inflammatory exudate containing "swirly" cells characteristic of P. haemolytica pneumonia. Calves euthanatized at 12 hrs post infection had similar but less severe lesions, which were associated principally with peribronchial alveoli. The lesions were most prevalent in the anterior and cardiac lobes and consisted of atelectasis, alveolar edema, congestion of alveolar vessels and a mixed inflammatory cell infiltrate accumulating principally in the alveoli and respiratory bronchioles. Calves that died at 6 hrs or less after challenge had scattered areas of

atelectasis accompanied by alveolar edema and an inflammatory response dominated by macrophages and neutrophils.

Data from these structural studies supports the hematologic and physiologic studies I described for the same groups of calves. Pasteurella haemolytica causes an acute pneumonia and bronchiolitis characterized by vascular damage, edema and mixed inflammatory cell infiltration. These structural alterations correspond well with the impairment of oxygen exchange, reduced dynamic compliance and with the rapid loss of neutrophils from the circulation, reflecting the injury of the pulmonary parenchyma as the initial lesion of pneumonic pasteurellosis.

INTRODUCTION

The gross and microscopic lesions associated with Pasteurella pneumonia of cattle were recently reviewed by Rehmtulla and Thomson (1981).[1] Although the lesions observed in lungs of severely ill cattle and those dying of pneumonia are well described, Rehmtulla and Thomson remarked upon the lack of information regarding the development of pulmonary lesions. One objective of the present study was therefore to describe the early lesions of pneumonic pasteurellosis and to correlate these findings with changes in lung function. The previous reports[2,3] describing early lesions were part of a study designed to examine mechanisms of bacterial clearance from calf lungs. Lesions described in some calves at 4 hours post inoculation consisted of alveolar edema and there was some evidence of increased numbers of mononuclear cells. Gilka et al (1974) postulated that the initial lesion of pulmonary pasteurellosis was pulmonary edema, which subsequently led to a washout of surfactant, atelectasis and subsequent bacterial overgrowth.[2,3] Tweed and Edington

in 1940 had speculated that lesions, assumed to be the earliest changes in naturally occurring cases of pasteurellosis, were those of alveolar congestion, edema and desquamation of epithelial cells.[4] However, others have suggested that atelectasis and alveolar edema are initially present and that Pasteurella then begin to grow in these particular areas.[5]

As reviewed by Rehmtulla and Thomson[1], the lesions of P. haemolytica pneumonia are thought to progress from atelectasis and pulmonary edema to those of acute fibrinous pneumonia and pleuritis, with areas of infarction and irregular foci of necrosis. There are, however, other reports of histologic lesions associated with pneumonic pasteurellosis that do not fit this classical description of Shipping Fever pneumonia; one of these lesions, described by a number of different authors as a prominent bronchopneumonia[4-10] associated with Pasteurella infection was thought uncharacteristic of the disease by Thomson[1], his coworkers[11] and others.[5,12,13] Clearly, there is conflicting evidence regarding the nature of lesions of experimental and naturally acquired pasteurellosis, even when the lesions are well developed. Differences in the lesions of experimental pasteurellosis may in part be due to differences in experimental design, since some methods of inducing pasteurellosis rely on previous or concurrent viral infections, but others do not.

This study was designed to describe the gross and microscopic lesions of acute P. haemolytica infection without concurrent viral exposure. Changes in pulmonary structure were correlated with those alterations in pulmonary function and blood constituents described in the previous chapters.

METHODS

Control Calves

Six neonatal calves (C1 through C6 respectively), instrumented and exposed to a single intratracheal injection of saline as previously described, were used as control animals. These animals had previously been chilled and acetic acid sprayed into the trachea (see previous chapters). After completion of the physiologic studies, animals were euthanatized by electrocution. The calves were promptly necropsied, with inspection of all organs, before samples of lung, liver and kidney were taken for microbiologic survey as outlined below. With the exception of brain tissue, which was fixed whole in 10% phosphate buffered formalin, and lung tissue, which was fixed in the manner described below, samples consisting of 1 cm thick slices of body organs were taken for routine histopathology. A major bronchus from the left and right apical, cardiac and diaphragmatic lobes was cannulated with a tightly fitting PE catheter and these lobes fixed by a combination of submersion and airway perfusion. Airway perfusion pressure was 33 cm of H_2O and the fixative was modified Karnovsky's fixative. The apparatus used for fixation is described in Appendix B. Sections of trachea and major bronchi were also fixed by submersion in Karnovsky's fixative. Sample sites for histopathologic evaluation are illustrated in Figure 4-1.

Pasteurella Group

Five neonatal calves (P1-P5), instrumented and exposed to a single intratracheal injection of 2×10^9 *P. haemolytica* organisms were euthanatized by electrocution 12 hrs after exposure to *P. haemolytica*. The sixth calf (P6) died at 18 hrs after challenge and a seventh calf (P7)

Figure 4-1. Schematic diagram of calf lungs illustrating the sampling sites for lung and airway tissue samples taken for histologic evaluation. Sample sites from the trachea and right mainstem bronchus are indicated by arrows. The lobes are labeled right apical (RA), right cardiac (RC), right diaphragmatic (RD), left diaphragmatic (LD), left cardiac (LC) and left apical (LA). The accessory lobe is not indicated and was not sampled.

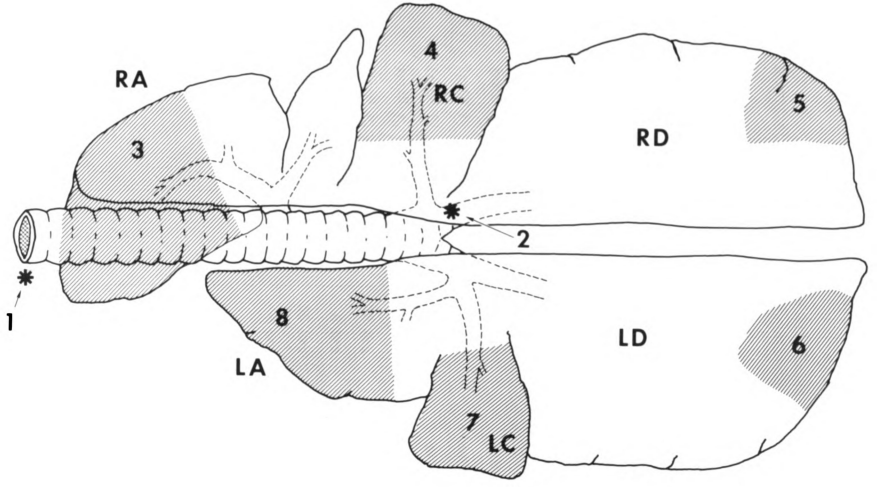

Figure 4-1

TABLE 4-1. Summary of experimental protocol for Control (C) and Pasteurella-exposed (P) calves

Calf	Bacterial Exposure	Time Till Death After Exposure	Manner of Death
C1	None	12 hrs	euthanatized
C2	None	12 hrs	euthanatized
C3	None	12 hrs	euthanatized
C4	None	12 hrs	euthanatized
C5	None	12 hrs	euthanatized
C6	None	12 hrs	euthanatized
P1	$2x10^9$ *P. haemolytica*	12 hrs	euthanatized
P2	$2x10^9$ *P. haemolytica*	12 hrs	euthanatized
P3	$2x10^9$ *P. haemolytica*	12 hrs	euthanatized
P4	$2x10^9$ *P. haemolytica*	12 hrs	euthanatized
P5	$2x10^9$ *P. haemolytica*	12 hrs	euthanatized
P6	$2x10^9$ *P. haemolytica*	18 hrs	died
P7	$2x10^9$ *P. haemolytica*	6 hrs	died
P8	$2x10^8$ *P. haemolytica*	36 hrs	died
P9	$2x10^9$ *P. haemolytica*	4 hrs	died

died at 6 hrs post challenge. Two further calves, not included in the physiologic studies, are also described. Calf 8 (P8) received 2 x 10^8 P. haemolytica organisms intratracheally and died 36 hrs after challenge. Calf 9 (P9) received 2 x 10^9 organisms intratracheally and died 4 hrs after exposure. The experimental protocol for all calves is summarized in Table 4-1. All calves in the Pasteurella group were necropsied and sections for microbiology and histopathology taken and processed in an identical manner to that described for control calves.

Microbiological Survey Methods

a. Viruses. Tissue samples were ground up using a mortar and pestle and placed in Eagles minimal essential medium[a] modified by the addition of 100 units/ml of crystallin penicillin G[b], 100 µg/ml of dihydrostreptomycin[c], 25 µg/ml of gentamicin[d], 5 µg/ml of fungazone[e], and volumes adjusted so that the final solution contained 10% of fetal bovine serum[f] and 2 m-molar glutamine.[g] The tissue suspension was filtered through a 0.45 µ membrane filter[h] and 0.2 µ liter aliquots of the filtrate inoculated onto glass coverslips containing monolayers of bovine turbinate cells.[i] Monolayers were examined daily for cytopathogenic effects up to a total of 7 days. If no characteristic

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a Grand Island Biological Co, Grand Island, NY.
b Pfizer Laboratories Division, Pfizer Inc., New York, NY.
c Pfizer Laboratories Division, Pfizer Inc., New York, NY.
d Schering Corporation, Kenilworth, NJ.
e ER Squibb and Sons, Inc., Princeton, NJ.
f Hyclone, Sterile Systems Inc., Logan, Utah.
g Grand Island Biological Co., Grand Island, NY.
h Type LS filter, Nalge Company, Division of Sybron Corporation, Rochester, NY.
i Supplied by the National Animal Disease Center, Ames, Iowa.

cytopathogenic effects were noted, monolayers were incubated with fluorescein labeled antibody for infectious bovine rhinotracheitis (IBR), bovine virus diarrhea (BVD), parainfluenza 3 (PI_3) and rotavirus. No conjugate was available for bovine adenovirus. Cultures that had no cytopathogenic effects at 7 days post inoculation were stained with Giemsa stain and examined for the presence of typical adenoviral inclusion bodies.

b. Bacteria. Samples from lung, liver and kidney were inoculated onto sheeps blood agar (SBA) plates[j], MacConkeys agar[j] plates and into selenite broth.[k] Inoculums were incubated at 37°C for 24 hrs. Positive cultures were determined to be either gram positive or gram negative and bacteria identified by routine bacteriologic diagnostic methods. Gram negative organisms not belonging to the Enterobacteriacea were inoculated onto TSI slants[j], Tryptose agar slants[j], MacConkey agar slants[j], Heart Infusion broth[j], nitrate[j], motility[l], citrate[m] and mannitol broths.[j] Bacteria that had a positive test on MacConkey agar were fermentative and were positive for catalase, nitrate, indole, oxidase and mannitol but negative for oxidation, urea, motility and citrate were considered characteristic of P. haemolytica.

Histopathology

Sections of all fixed tissues were embedded in paraffin[n], stained with hematoxylin and eosin and examined by light microscopy. Samples

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j Difco Laboratories, Detroit, Mich.
k Selenite F broth, BBL Division, Becton, Dickinson and Co., Cockeysville, MD.
l MIO medium, Difco Laboratories, Detroit, Mich.
m Simmons Citrate, Difco Laboratories, Detroit, Mich.
n Paraplast, Division of Sherwood Medical, Brunswick Co., St. Louis, Mo.

from the respiratory tract were processed in the same manner except that paraffin embedded tissues were thin sectioned at 3 to 4 μ rather than 6 to 7 μ used for other tissues. In addition, samples of the respiratory tract were embedded in glycol methacrylate[o], sectioned at 1 to 2 μ using glass knives and stained with toluidine blue for examination by light microscopy.

RESULTS

1. Microbiologic survey. No evidence of viral infection was found in any calf. Samples of lung tissue from control calves C1 through C6 had light to moderate mixed bacterial growth of a variety of species, including Escherichia coli, alpha hemolytic Streptococcal sp, Pasteurella haemolytica, Bacillus sp, Micrococcus sp, Staphylococcus saprophyticus and Serratia liquefasceins. Calves P2 and P4-P8 had pure cultures of P. haemolytica isolated from the lungs and calves P6 and P8 had P. haemolytica also isolated from liver and mediastinal lymph node. Calves P1, P3 and P9 were negative for P. haemolytica and had light growths of Pseudomonas aeroginosa, alpha haemolytic Streptococcus sp, Escherichia coli and Acinetobacter sp. Microbiologic specimens from calves P1, P2, P3 and P9 were stored at 4°C for 36 hours prior to culture.

Gross Pathologic Lesions

Calves from the control group had a severe, focal necrotizing tracheitis, with accumulation of necrotic debris in the tracheal lumen.

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o Polysciences Incorporated, Paul Valley Industrial Park, Warrington, PA.

The lesion was situated at the level of the thoracic inlet and extended several centimeters proximally and distally. (Figure 4-2). Large airways distal to this focal lesion appeared normal and the trachea proximal to the tracheostomy site, the pharynx, larynx and nasal cavity were also of normal appearance. The lungs of all control calves were of normal consistency. Most of the lungs had a normal appearance on both pleural and cut surfaces but in all calves a few isolated lobules appeared atelectatic; these areas had no particular distribution throughout the lungs and were characterized by irregular polygonal areas which were of deep reddish-purple color of about 1 cm width and when appearing on pleural surfaces were depressed below the level of the remaining lung surface. (Figure 4-3). Gross lesions were not noted for any other tissues in control calves.

Calves exposed to P. haemolytica that were examined from 6 to 18 hrs post exposure had very similar gross pathologic changes compared to each other. Focal necrotizing tracheitis with accumulations of necrotic mucosa and exudate were present in all calves at the site of acetic acid exposure. There were no lesions noted in the nasal cavity, the pharynx, larynx or major bronchi and trachea distal to the thoracic inlet. The lungs had numerous areas of reddish-gray to reddish-purple discoloration. (Figure 4-4). On cut surface these areas were often centered around 3-5 mm diameter bronchi but were not exclusively found in these peribronchial areas. (Figure 4-5). The lesions appeared most numerous in the anterior lobes and the ventral aspects of the cardiac lobes but were also found in accessory and diaphragmatic lobes. Observed from the pleural surface, the lesions were depressed below surrounding lung tissue and in the apical lobes often coalesced so that

Figure 4-2. Focal tracheitis resulting from acetic acid exposure. Trachea of calf C3 with necrosis of the mucosa and accumulation of necrotic exudate on the luminal surface. The tracheostomy site (arrow) lies at the top of the page

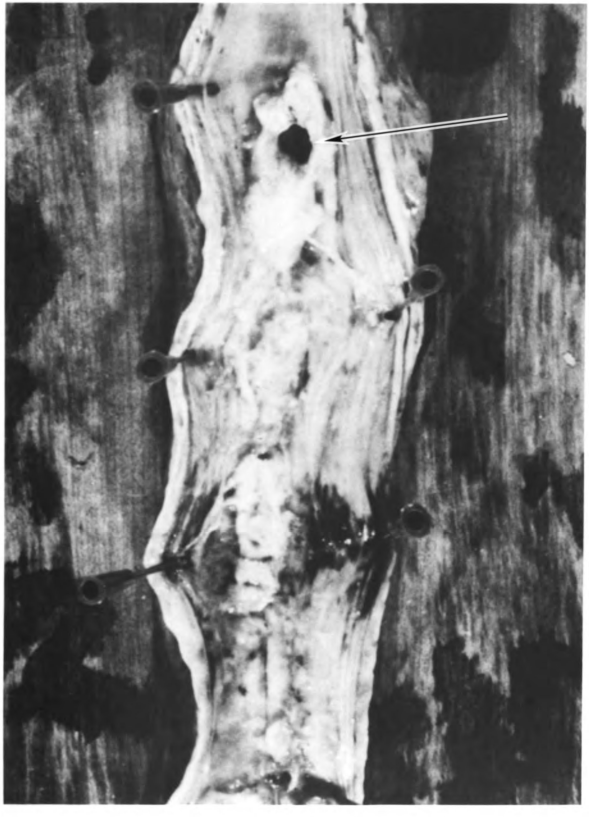

Figure 4-2

Figure 4-3. Macroscopic appearance of normal calf lungs. The lungs are from calf C5. The lungs are normal with the exception of several small areas of atelectasis that appear as darker depressed regions (arrows). The trachea lies toward the top of the page. The marker is 15.5 cm in length.

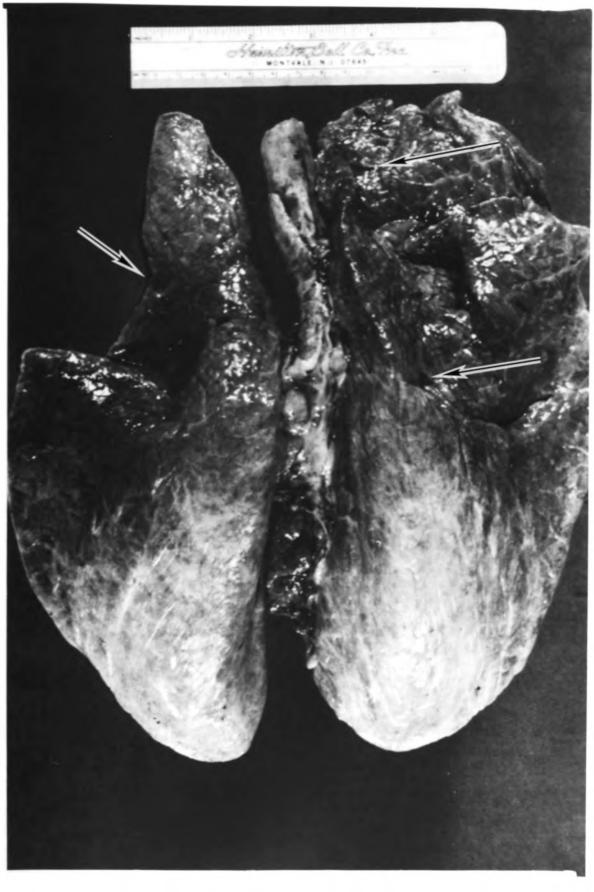

Figure 4-3

Figure 4-4. Macroscopic appearance of pneumonic Pasteurellosis in a calf. The lungs are from calf P5. The dark areas are areas of pneumonia and are concentrated in the right cardiac, both apical and the right diaphragmatic lobes. The trachea lies toward the top of the page. The marker is 15.5 cm in length.

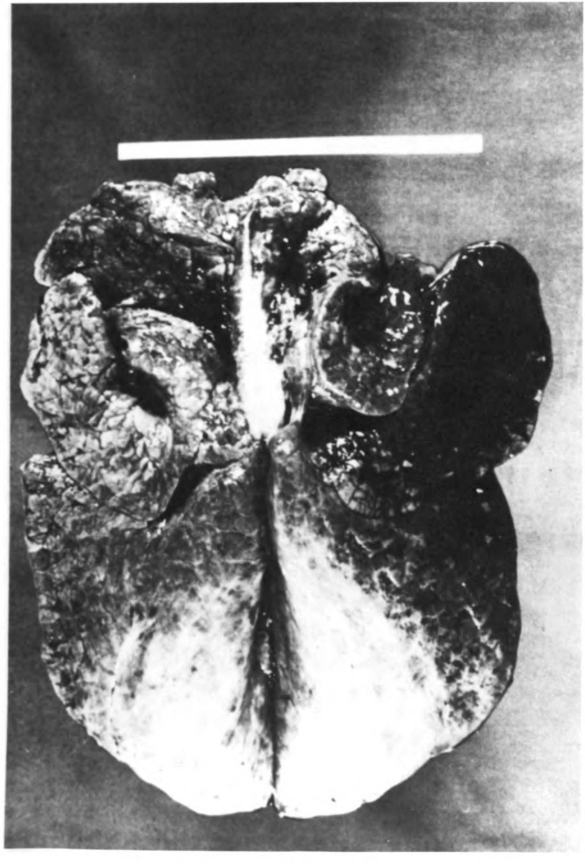

Figure 4-4

Figure 4-5. Subgross micrograph of lungs affected with pneumonic Pasteurellosis. The lesions are most severe in lobules closest to the major bronchi. Lung section from the right apical lobe of calf P11. Exudate is present in only one bronchus of the section (arrow) and the interlobular septa are widely dilated with fluid. Giemsa stain. Marker indicates 1 cm.

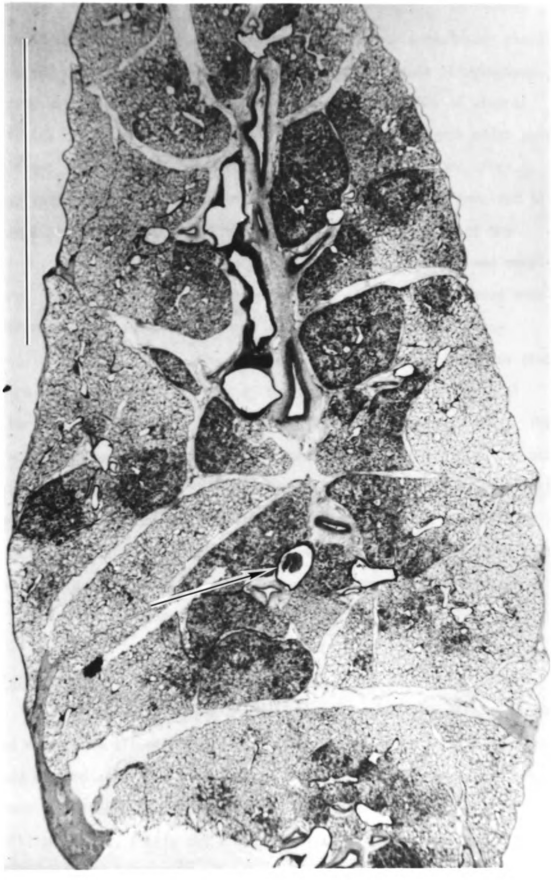

Figure 4-5

considerable numbers of adjacent lobules were affected. Sectioning through these lesions resulted in the oozing of serosanguineous fluid from the cut surface. An estimated 10 to 15% by volume of pulmonary tissue was affected in each calf. There was no evidence of pleural effusion or pleuritis in these calves but mediastinal lymph nodes were enlarged, congested and edematous. Calf P8 (died at 36 hrs after P. haemolytica inoculation) had an estimated 50% of the lung involved in a severe pneumonic process. There was extensive involvement of the anteroventral aspects of the lungs which were red to purple and very firm. The pleura over the affected regions of lungs was covered with a fibrinopurulent exudate and there was excessive amounts of serosanguineous fluid in the pleural cavity which contained fibrinous clots. There were fibrinous adhesions of the visceral pleural to parietal pleura in the anteroventral regions of the thorax. (Figure 4-6). The trachea and lymph nodes had changes similar to those described for other Pasteurella exposed calves. Calf P9 (died at 4 hrs after P. haemolytica inoculation) had changes in the trachea associated with tracheostomy and exposure to acetic acid but the lungs were not different to those in the control group. No other organs were abnormal on gross examination of all Pasteurella exposed calves.

Histopathology

The severe damage to the tracheal mucosa caused by acetic acid spray was evident in all calves. Histologic sections taken through the acetic acid exposed area were devoid of mucosa. The luminal surface of these areas was lined by a diptheritic pseudomembrane consisting of necrotic cellular debris, fibrin and a neutrophilic infiltration. The

Figure 4-6. Extensive pneumonia and pleuritis in a calf infected with P. haemolytica for 36 hrs (calf P8). There is extensive pneumonia involving all but the dorsal aspect of the diaphragmatic lobes. The trachea lies toward the right. The pericardial sac is visible (single arrow) and a large fibrinopurulent adhesion lies on the pleural surface of the right diaphragmatic lobe (double arrows).

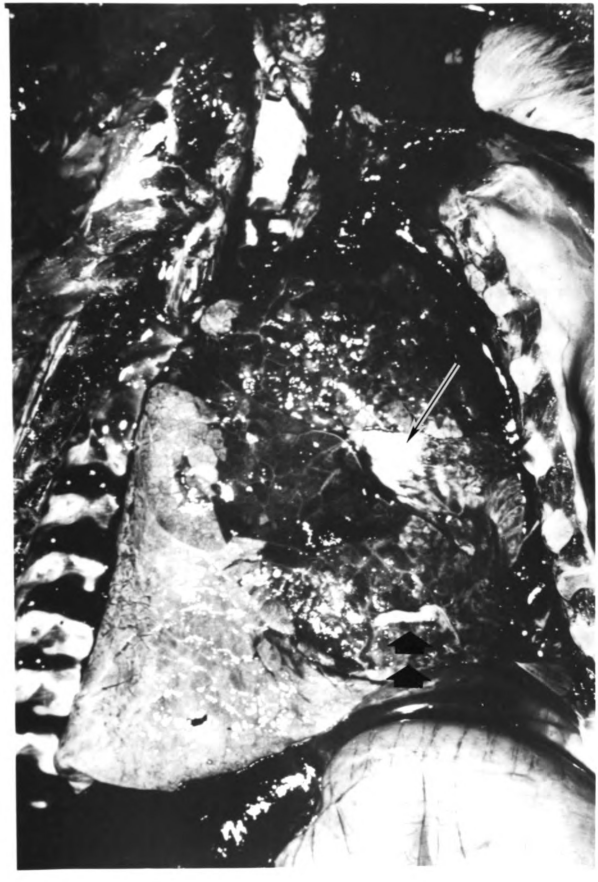

Figure 4-6

superficial layers of underlying submucosa were also necrotic. The remaining submucosa was edematous and infiltrated with neutrophils. (Figures 4-7). The lesion was limited to the trachea. The mucosal surfaces of mainstem bronchi and their ramifications were normal. However, relatively small numbers of airways and alveoli had accumulations of necrotic exudate, often mixed with sloughed pseudostratified epithelium, present in their lumens. Occasionally these masses completely obstructed airways and were associated with small areas of atelectasis (Figure 4-8).

The majority of pulmonary tissue in the control calf sections was normal. Because of the pressure-perfusion method of fixation, lung tissue was expanded. (Figure 4-9). Sections of lung tissue prepared in this manner had widened zones of connective tissue separation around blood vessels, bronchi and interlobular interstitium. (Figure 4-10). A minor proportion of lobules from lungs of all control calves were abnormal. These appeared partially collapsed, had increased numbers of mononuclear cells free in the alveolar spaces and in the most severe instances had accumulations of neutrophils within alveolar spaces. In these lobules the alveolar walls were congested and occasionally small areas of hemorrhage and accumulation of proteinaceous material were noted in alveolar lumens and terminal airways. (Figure 4-11).

Microscopic lesions were noted in the livers and small intestines of control calves. Sections of liver tissue from all control calves had periportal accumulations of mixed inflammatory cells. With the exception of calves C2 and C6, the liver changes were also accompanied by small necrotizing focal lesions in the parenchyma which had a

Figure 4-7. Micrograph of the tracheal lesion of a calf following acetic acid spray into the tracheal lumen. The necrosis of tissues extended into the submucosa and stimulated an intense infiltrate with inflammatory cells consisting principally of neutrophils (inset). Calf C4, H&E stain. The marker indicates 3 mm.

Figure 4-7

Figure 4-8. Micrograph of lung tissue from a control calf illustrating focal areas of atelectasis associated with bronchial obstruction. The material obstructing the terminal bronchi consists of neutrophilic exudate and sloughed sections of pseudostratified respiratory epithelium (arrows) and is shown in detail in the inset. Left cardiac lobe of calf C6, H&E stain. Marker indicates 1 cm.

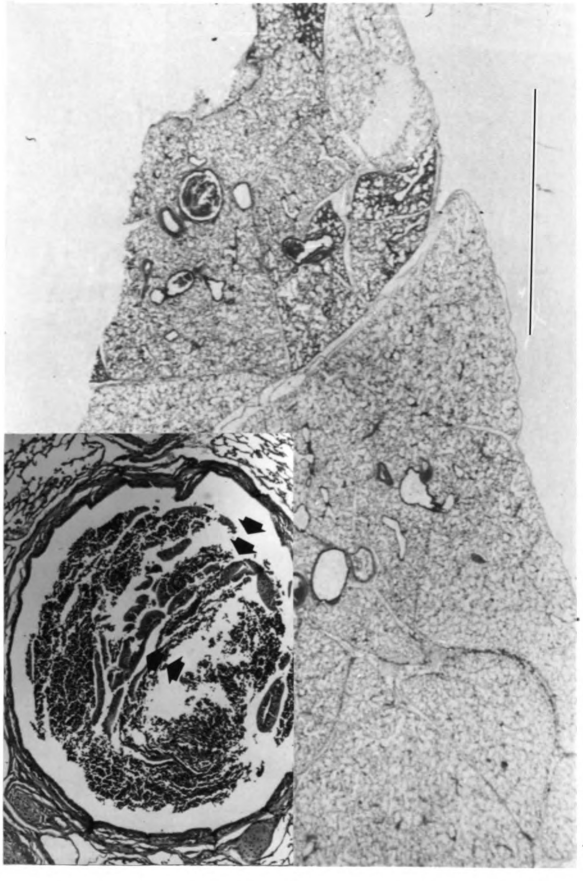

Figure 4-8

Figure 4-9. Effect of lung fixation by perfusion of airways with fixative under pressure on normal calf lungs. Right diaphragmatic lobe of Calf C4. H&E stain. Marker indicates 5 mm.

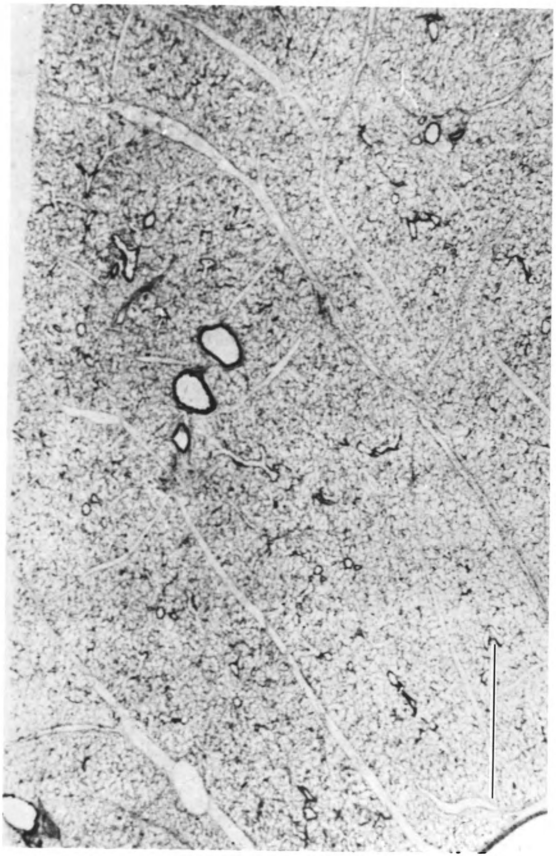

Figure 4-9

Figure 4-10. Micrograph of normal calf lungs illustrating "edema" artifact resulting from airway pressure perfusion with fixative. Right diaphragmatic lobe of calf C4. H&E stain. Marker indicates 1 mm.

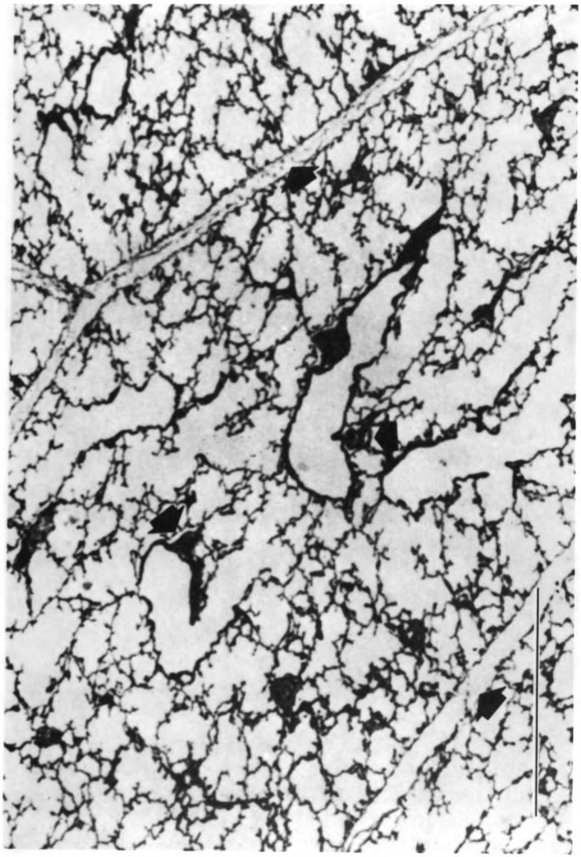

Figure 4-10

Figure 4-11. Photomicrograph of a focal lesion in the pulmonary parenchyma of a control calf. Alveoli are not fully inflated and their lumens contain inflammatory cells consisting mainly of neutrophils but also with occasional large mononuclear cells and erythrocytes. Calf C4, H&E stain, marker indicates 200 micron.

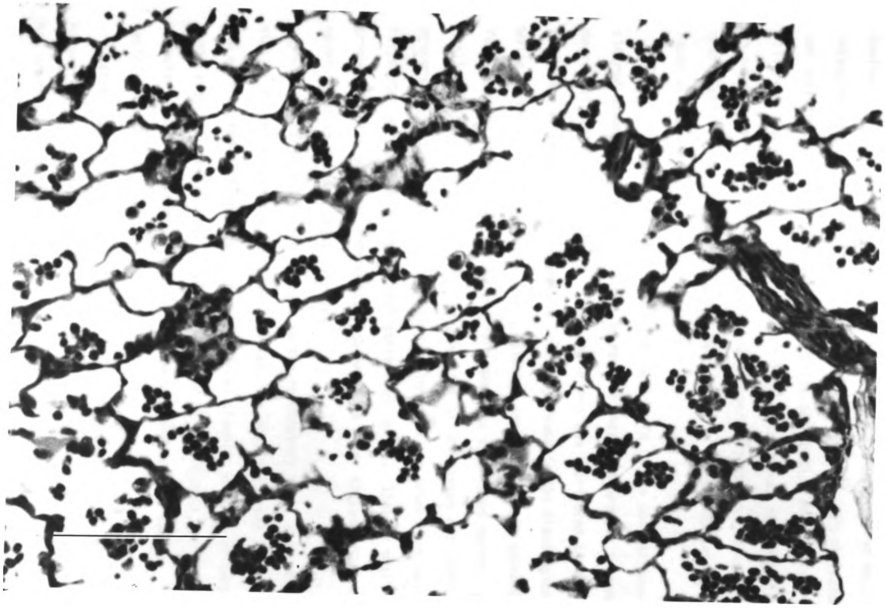

Figure 4-11

dominant neutrophilic infiltrate. (See Figure 4-24) Sections of small intestine from calves C4, C5 and C6 had areas where villi were shortened and occasionally fused. There was congestion of the tips of affected villi and a mild to moderate increase in lymphomononuclear cell numbers in submucosal areas. In all three calves, numerous protozoal forms characteristic for Cryptosporidium sp were noted on the epithelial surfaces of affected villi. (Figure 4-12).

The histologic appearance of control calf brain tissue was not considered abnormal, although on first impression there appeared to be some degree of perivascular cuffing. This appearance was attributed to the abundance of connective tissue nuclei normally found in the blood vessel walls of neonatal calves. Similarly, although the width of the splenic follicles was narrow (100 to 200 μm) this was also considered normal. Small clusters of cells, principally comprised of leukocytes and their precursors, but occasionally containing erythrocyte precursors and megakaryocytes were noted in the adrenal cortex and spleen. Similar small groups of cells were not infrequently observed in liver tissue but were never found to contain megakaryocytes.

Calves P1, P2 and P9 had changes in the respiratory tract that were similar to those observed in control calves. In addition, some lobules had mild to moderate amounts of hemorrhage and alveolar edema, and large numbers of inflammatory cells, often dominated by neutrophils, in the alveolar spaces and terminal airways. Affected lobules were atelectatic. (Figures 4-13 to 4-15).

Calves P3-P7 had more extensive and severe lesions than calves P1, P2 and P9. Major airways had normal mucosal surfaces but occasionally had necrotic cellular debris and inflammatory exudate plugging their

Figure 4-12. Photomicrograph of the small intestine of a calf with subacute enteritis associated with Cryptosporidium sp. Villi are shortened and there is a lymphomononuclear infiltrate in the submucosa. At greater magnification (inset) numerous protozoan forms can be visualized on the mucosal cell surfaces (arrows). Ileum of calf C4, H&E stain, marker indicates 10 micron.

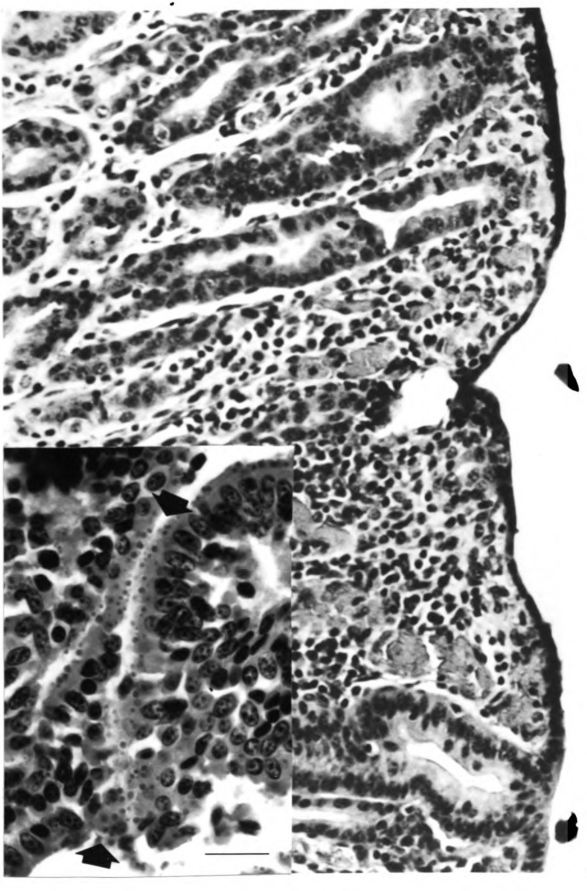

Figure 4-12

Figure 4-13. Photomicrograph of the histologic lesions of lungs infected with Pasteurella haemolytica. A bronchiole containing a small amount of purulent exudate is situated near the center of the micrograph. Alveoli are only partially inflated and contain cellular exudate consisting mainly of neutrophils. Some alveoli are filled with protein rich, edema fluid (arrows). Calf P2, H&E stain, marker indicates 0.2 mm.

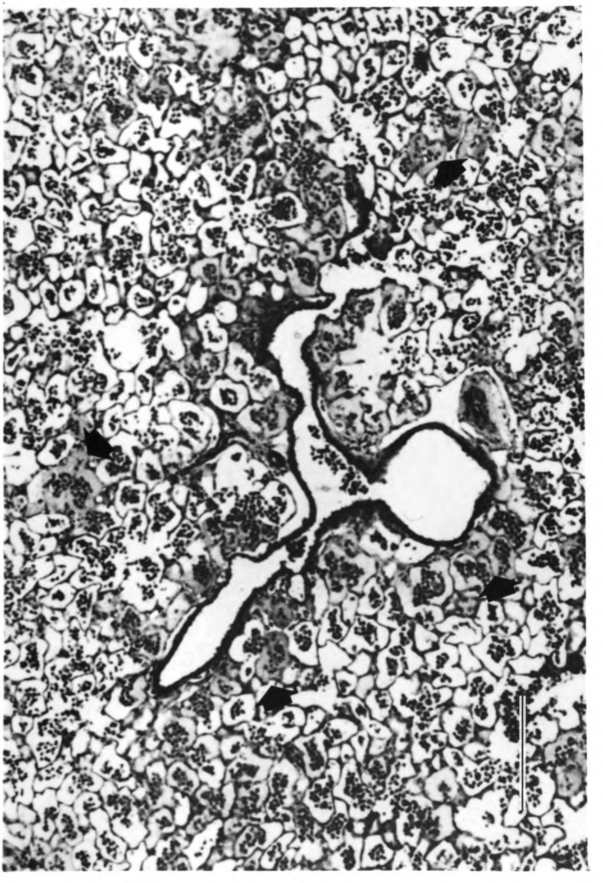

Figure 4-13

Figure 4-14. Photomicrograph of a necrotizing alveolar lesion from a calf infected with Pasteurella haemolytica. Alveolar wall necrosis, edema and hemorrhage is present in the center of the micrograph. A mild neutrophilc inflammatory response is present. Calf P2, H&E stain, marker indicates 0.5 mm.

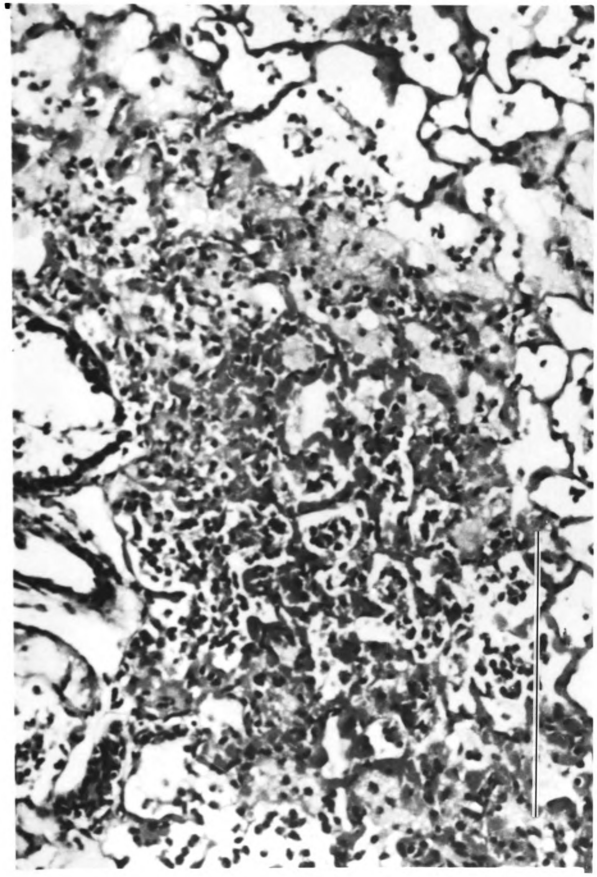

Figure 4-14

Figure 4-15. Photomicrograph of lymphatic distension with edema associated with pneumonic Pasteurellosis. Interstitial and perivascular edema is present and lymphatics are distended with a cell free protein rich material (arrows). Calf P2, H&E stain, marker indicates 0.5 mm.

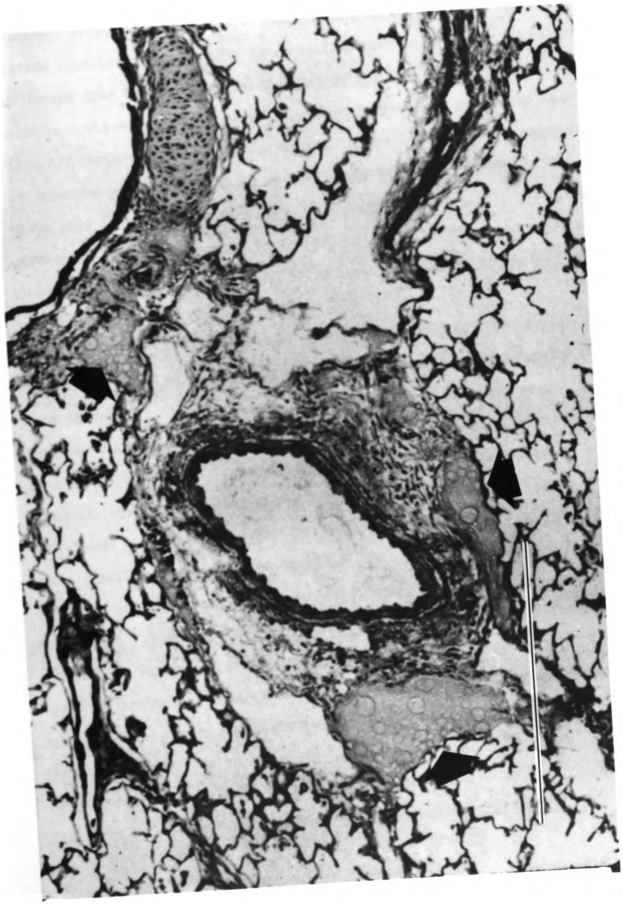

Figure 4-15

lumens. (Figures 4-16to 4-19). The bronchioles and alveolar ducts contained exudate consisting of a mixed inflammatory cell exudate quite different than that observed in the control calves. (Figure 4-20). The exudate contained numerous basophilic degenerative cell cytoplasms that also had large smudged basophilic nuclei. There were also large numbers of degenerating neutrophils and bacteria, the latter being readily visualized as small short rods on Giemsa stains (Figure 4-21), and which were gram negative. Many of the mononuclear cells in the exudate had a characteristic fusiform, swirling or streaming appearance to their cytoplasms.

The alveolar walls of affected lobules had congested blood vessels but accumulations of cells, exudate or thrombi within alveolar walls were not noted. Alveolar lumens nearest major airways seemed most severely involved, but atelectasis of alveoli in otherwise unaffected lobules was also noted. Alveoli often contained much proteinaceous material, with variable amounts of hemorrhage and exudate similar to that found in the bronchioles. In some areas the proteinaceous material had a fibrillary network, suggestive of fibrin. In some areas, where there was obvious alveolar wall necrosis, there was extensive hemorrhage. (See Figure 4-19). The development of exudate in alveoli and of proteinaceous fluid deposition with or without hemorrhage appeared to occur as independent processes as some alveoli had cellular exudate alone, others had edema alone and yet both occurred together in the majority of alveoli. (See Figures 4-13, 4-14 and 4-16) There were focal areas of subpleural and peribronchial emphysema in areas of lung that were not affected with pneumonia. (Figure 4-22)

Figure 4-16. Photomicrograph of lobular pneumonia in a calf exposed to Pasteurella haemolytica. The lobule contains areas of intense cellular infiltrate and others where alveolar edema is the principle lesion. Interstitial lymphatics are distended with edema fluid (arrow) and neighboring lobules are atelectatic. Calf P6, H&E stain, marker indicates 1 cm.

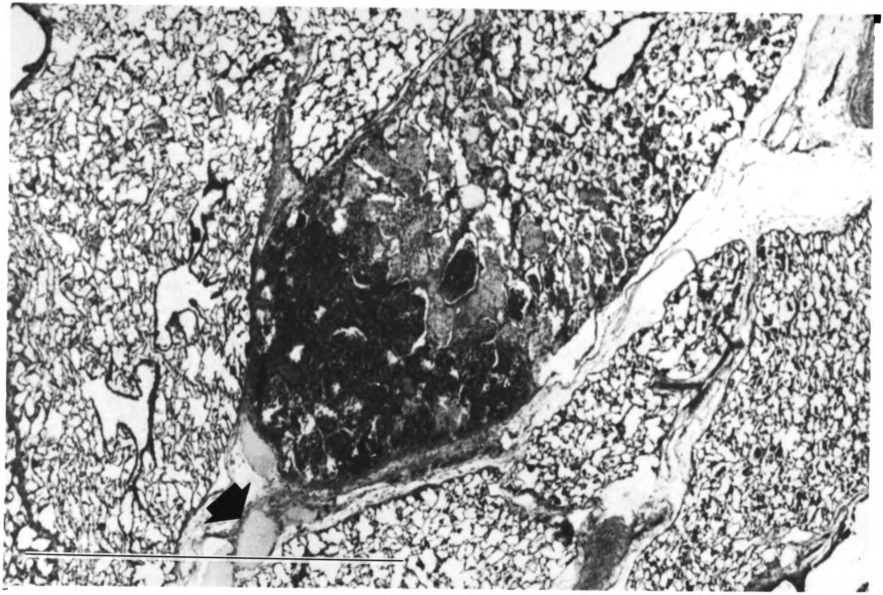

Figure 4-16

Figure 4-17. Photomicrograph of well developed pneumonic lesions in a calf inoculated with Pasteurella haemolytica. The lobule is extensively infiltrated with inflammatory cells and exudate has accumulated in the larger airways. Calf P6, H&E stain, marker indicates 0.5 mm.

Figure 4-17

Figure 4-18. Photomicrograph of well developed pneumonic lesions in a calf inoculated with Pasteurella haemolytica. Alveolar tissue has collapsed and extensive cellular exudate has accumulated in the lobules. The interstitial lymphatics are greatly distended with edema fluid and contain large fibrinous thrombi that have entrapped inflammatory cells (arrows). Calf P6, H&E stain, marker indicates 0.1 mm.

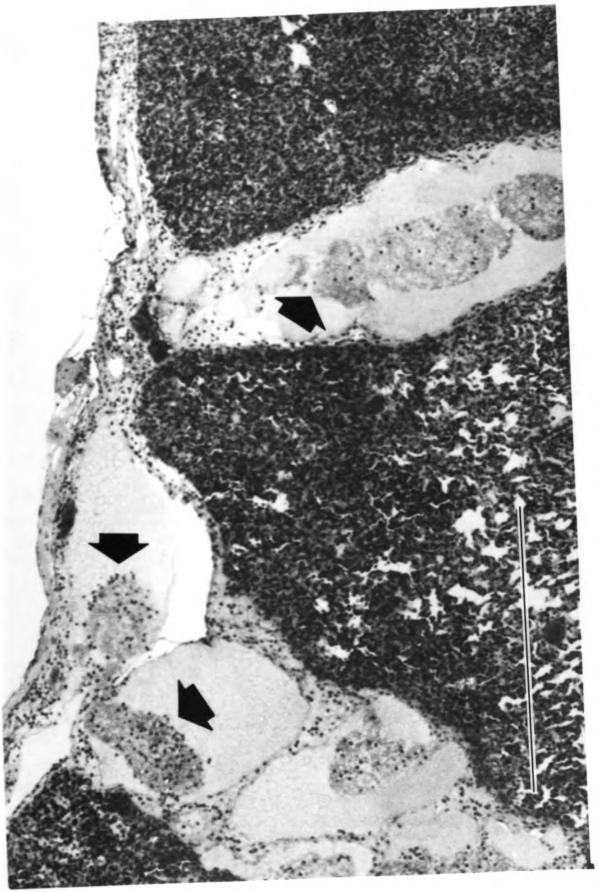

Figure 4-18

Figure 4-19. Photomicrograph of acute necrotizing lesions in the pulmonary parenchyma of a calf inoculated with Pasteurella haemolytica. Alveolar walls are no longer distinguishable, the structures remaining principally consisting of necrotic debris, inflammatory cells, erythrocytes and edema fluid. Calf P7, H&E stain, marker indicates 0.1 mm.

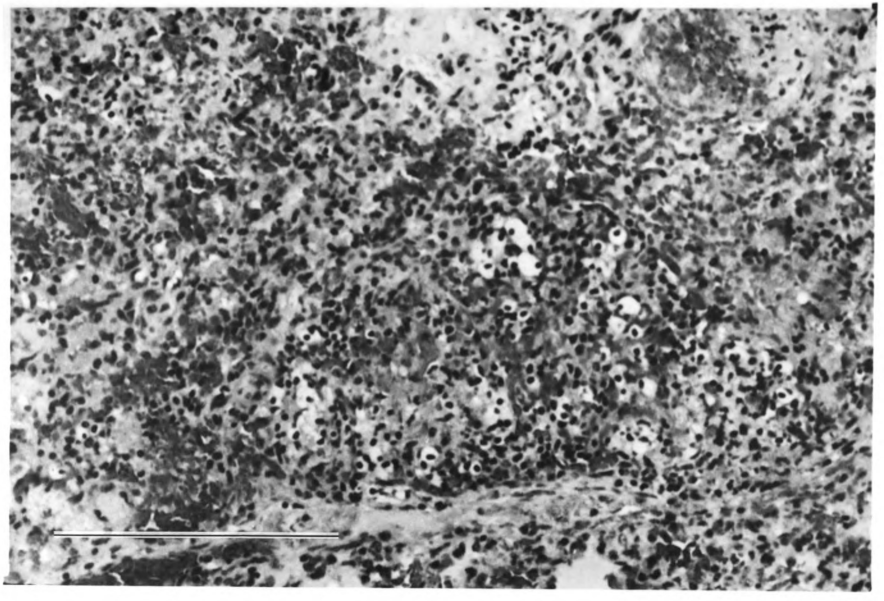

Figure 4-19

Figure 4-20. Comparison of the histologic appearance of bronchiolar exudates of control and Pasteurella-exposed calves. Figure 4-20A indicates typical exudate from control calf studies (C4) and consists mainly of neutrophils. Figure 4-20B illustrates the exudate associated with well developed lesions of pulmonary pasteurellosis (P5) and consists of mononuclear cells, many of which are smudged (arrows). There is some suggestion of a streaming pattern. Marker indicates 20 microns.

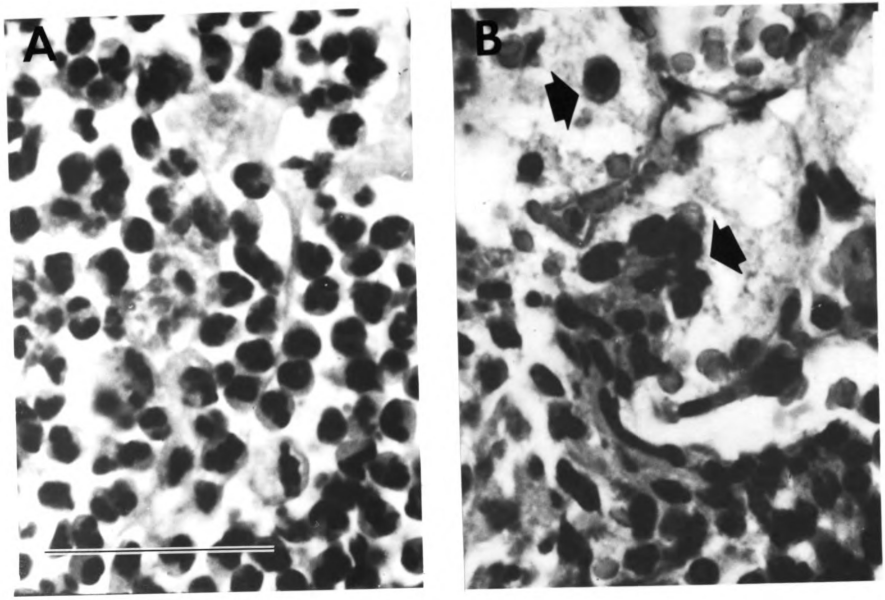

Figure 4-20

Figure 4-21. Photomicrograph of the inflammatory response to Pasteurella haemolytica for an established pulmonary lesion. Streaming of indistinct mononuclear cells is apparent and numerous bacteria are present in the exudate accumulating in alveolar lumens. Calf P5, Giemsa stain, marker indicates 10 microns.

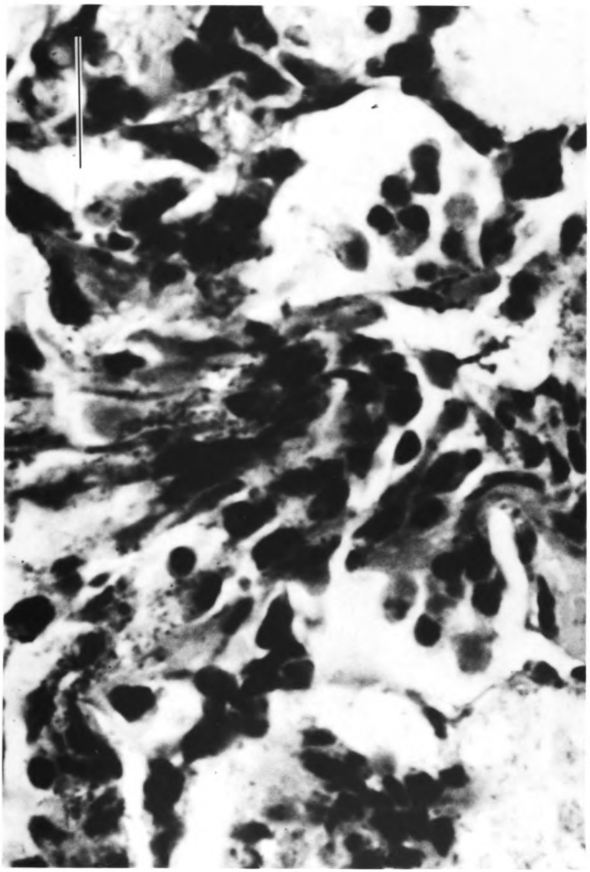

Figure 4-21

Figure 4-22. Photomicrograph of subpleural and peribronchiolar hyperinflation in otherwise normal lung tissue of a calf inoculated with Pasteurella haemolytica. In the most severe areas of overinflation, rupture of alveolar walls has occurred. Calf P5, H&E stain, marker indicates 1 mm.

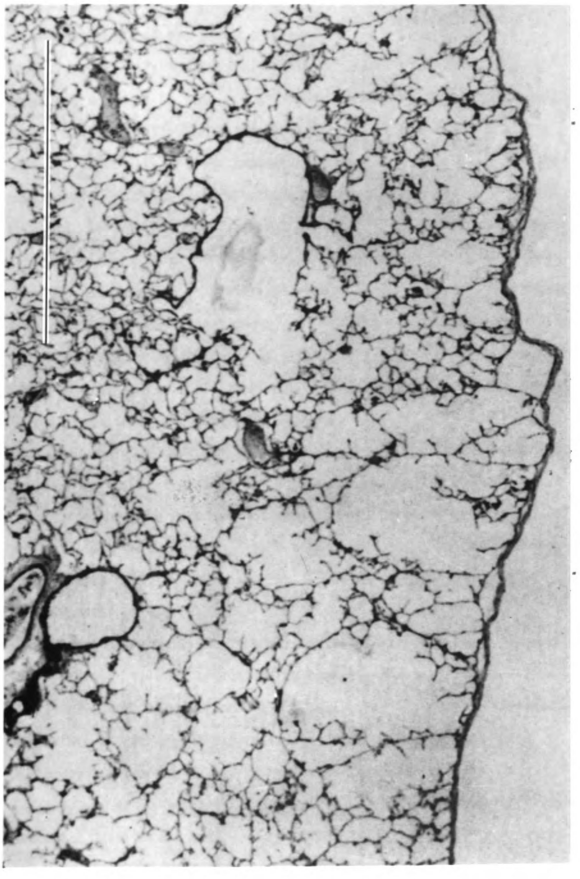

Figure 4-22

There were no lesions in the arterial vasculature but veins were often congested and had pronounced margination of leukocytes, principally neutrophils against endothelial surfaces. Capillaries were congested not only in lobules containing exudate but were also congested in the alveolar walls of neighboring lobules. The lymphatics associated with airways and the interstitium were distended with proteinaceous fluid but only rarely contained inflammatory cells or fibrinous thrombi. The pleural surfaces of the lungs had similar inflammatory changes as a result of extension from neighboring lobules. Fibrinous thrombi containing small numbers of inflammatory cells were present on the pleural surfaces of pulmonary sections from P6. (See Figure 4-18).

All calves in the Pasteurella group had a neutrophilic inflammatory response of the bronchial lymph nodes, with large numbers of neutrophils filling medullary and subcapsular sinuses. The lymph nodes were congested and edematous and in calves P2, P4 and P7, the lymphoid tissue in the cortices of the nodes was depleted. (Figures 4-23).

Calf P8 had the most severe lesions, with large irregular areas of necrosis that were often confluent between lobules. The inflammatory changes were histologically similar in nature to those previously described for calves P3-P7.

Calves in the Pasteurella group had a similar histologic appearance of hepatic and splenic tissue as the control calves. (Figure 4-24). Calves P2 and P3 had similar changes in the small intestine as described for calves C4, C5 and C6 except that no protozoal organisms were observed. Calves P1, P2 and P3 had mild lymphomononuclear to mixed inflammatory cellular perivascular cuffing in the brain and in P2 this was associated with a single microabscess in the cerebral gray matter.

Figure 4-23. Acute suppurative lymphadenitis of mediastinal lymph node in a calf inoculated with Pasteurella haemolytica. The gland is edematous with dilation of medullary and subcapsular sinuses which contain numerous neutrophils (see inset). Depletion of cortical lymphoid tissue is pronounced (arrows). Calf P6, H&E section, marker indicates 5 mm.

Figure 4-23

Figure 4-24. Inflammatory changes in the liver of a calf illustrating focal areas of necrosis and periportal inflammation. The focus of necrosis had an intense inflammatory reaction, comprised predominantly of neutrophils, in the liver parenchyma. A mixed inflammatory cell response is present in the periportal area of the lobule (arrow). Calf P2, H&E stain, marker indicates 0.1 mm.

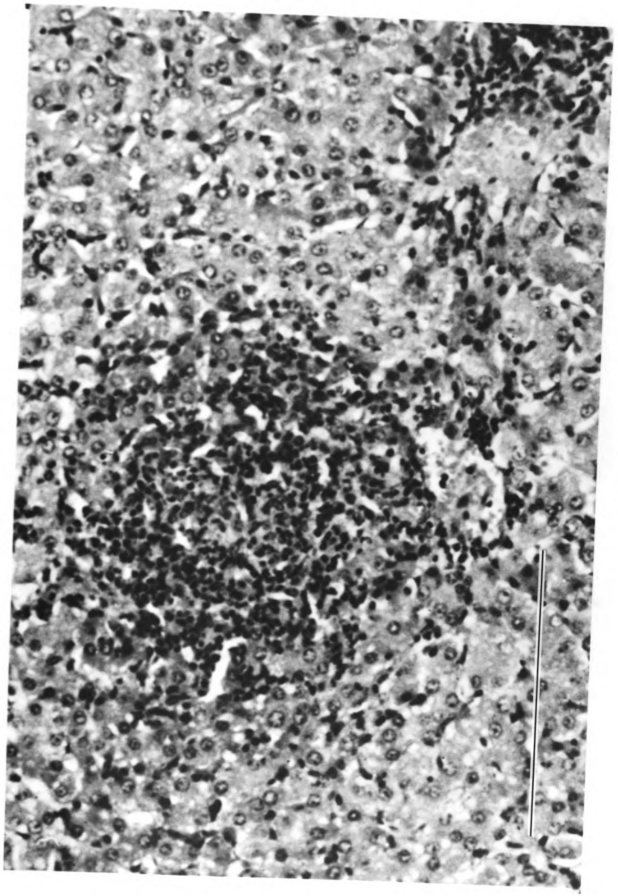

Figure 4-24

A single thrombus was noted in a small arteriole in the brain of calf P3. Bacteria were not observed in the perivascular cuffs or in the microabscess using H&E, Geimsa and Gram stains. Sections of pituitary, kidney, heart, adrenal, spleen, pancreas, skeletal muscle, tongue and colon were normal in all calves.

Sections embedded in glycol methacrylate had little evidence for alteration in mast cell numbers or their degree of granulation, or for changes in the numbers of goblet cells and the staining properties of their mucus.

DISCUSSION

The respiratory system of the control group of calves was clearly abnormal structurally but was not functionally affected because of the mild nature of the lesions. (See chapter 2). The pulmonary lesions of control calves consisted of atelectasis and foci of edema and neutrophilic inflammation. Several influences may have invoked this reaction. The acetic acid spray was obviously a potent necrotizing agent in the trachea, where presumably most of the chemical was delivered. The potential for aerosolized droplets to be inhaled into the lungs was present and these may be in part responsible for the lesions observed. In previous studies, that used acetic acid as part of a protocol to infect calves with Pasteurella spp, adverse effects of acetic acid on the respiratory system were not noted except in one calf[14,15], that reportedly developed tracheal ulceration after submucosal injection with acid. However, acetic acid may have contributed to the histologic changes noted in the lungs.

Other than a direct effect of acetic acid, inhalation of necrotic mucosa, exudate and neutrophils which originate from the acetic-acid-induced tracheal lesion may lead to small airway obstruction, atelectasis and local inflammatory changes in the lungs.

As suggested in the preceeding chapters, cold stress may facilitate the development of pneumonia by immune suppression and increased exposure of alveolar surfaces to inspired pathogens. Isolation of small numbers of mixed bacterial types was possible from the lungs of all control calves. These small mixed bacterial infections may have contributed to the development of the pulmonary lesions of control calves. Despite the lesions found in control calves, whatever their cause, we believe that the animals fulfilled the role of control animals, since the majority of lung tissue remained normal and respiratory system function remained unimpaired.

A structural change occurred in all lung tissues of control (and Pasteurella) calves unassociated with any antemortem tissue injury. This change is the so called "edema artifact" induced by pressure perfusion of the airways with fixative and explains the widening of interstitial, peribronchial and perivascular tissue spaces with fixative fluids.[16] Antemortem edema of these regions is therefore very difficult to assess unless accompanied by a high protein content. The advantage of lung samples prepared in this manner, was that the size and state of inflation of alveoli could be critically assessed. Previous studies of pneumonic pasteurellosis have not prepared pulmonary tissue in a standardized manner suitable for assessing alveolar sizes.

The aging of acute pulmonary lesions based on histological appearance is difficult and yet, to date, understanding of the mecha-

nisms of P. haemolytica induced pulmonary injury have relied on such interpretations. Exceptions are the studies by Gilka et al (1974)[3,4] and Friend et al (1977)[11] who recorded the length of time post exposure to P. haemolytica till death, during the first days of P. haemolytica pneumonia. Hallmarks of established lesions in other organs may not be valid in lungs since blood, fluid and macrophages may be present in alveoli in early as well as developed lesions. Other difficulties with histologic interpretation of pulmonary tissues that have, to date, been ignored are the possible presence of concurrent subclinical disease and the failure to fix lung specimens under standardized conditions.

In this study I minimized these detrimental influences on histologic interpretation by using neonates tested for pulmonary function as subjects (minimizing the risk of concurrent or chronic disease) and by fixing the lungs in a standardized manner. I also avoided the rationalization that less severely affected lobules reflected early changes, since there is no evidence to the contrary to indicate that such lesions are not simply a reflection of graded responses to injury.

From physiologic studies (see Chapter 3) it was predicted that the early lesions of bovine pneumonic pasteurellosis would be associated with pulmonary parenchyma and peripheral airways. The lesions of P7 and P9 examined at 4 and 6 hrs post challenge respectively indicated the initial involvement of parenchymal tissues without changes in the major intrapulmonary airways. Similar changes are described in calves 4 hrs after exposure to P. haemolytica.[3]

The spread of inflammatory exudate into small bronchi had occurred by 12 hrs post exposure. In my study, involvement of the major airways

either by plugging with exudate or by necrosis and inflammation of the bronchial walls was not a feature of the disease, even in the animal that lived for 36 hrs. In other studies significant bronchitis was absent in calves exposed to P. haemolytica aerosol for at least 18 hrs[2,3,11] but is present in experimental cases after three[11] or four[8] days.

One aim of this project was to determine alterations in alveolar lumen dimensions and alveolar wall thickness in calves 12 hrs after infection with P. haemolytica. I did not anticipate the extent and severity of lesions found after this time, as they were similar to well developed lesions described in naturally occurring cases and experimental cases that were several days old.[1,3,8,11] Because of these severe changes, a morphometric analysis of alveolar sizes had to be abandoned since many areas had extensive necrosis of alveolar boundaries. The characteristics of pneumonia by 18 hrs post infection were well summarized by Rehmtulla and Thomson (1981)[1] who describe interstitial and alveolar edema, interstitial lymphatic thrombosis, fibrinous pleuritis and alveolar wall necrosis. From the data of calf leukograms, where pronounced neutropenia developed, and the pulmonary histologic studies, it is evident that neutrophils have a significant role in the disease both in the early and developed responses to P. haemolytica.

I describe leukopenia in calves associated with P. haemolytica pneumonia as has been described elsewhere[8,17], and found suppurative lymphadenitis and neutrophilic exudates in alveolar spaces and bronchioles of calves examined histologically 12 hrs after exposure. I therefore differ from the view of Rehmtulla and Thomson[1], that suppurative responses are atypical of the disease. Others have also noted

exudates containing many neutrophils in cases of experimental P. haemolytica pneumonia in calves.[8,9,11]

Mononuclear cells have been described by Gilka et al (1974) as the initial inflammatory cell involved in the responses to P. haemolytica exposure. My data suggests that mononuclear cells, principally macrophages, have a role in both early and developed pulmonary lesions, but that this response is frequently mixed with neutrophilic infiltrates. Thomson and others have attributed the "swirling" or "streaming" basophilic mononuclear cells, so characteristic of bovine pasteurellosis, to altered populations of macrophages. I cannot confirm that macrophages are the only cell type to contribute to the exudate which contains swirly cells. Degenerative changes in the alveolar type II cells have been observed, resulting in their sloughing into the cellular debris collecting in alveolar lumens.[1,3,18] Other mononuclear cells such as bronchiolar cells, and migrating lymphoid cells might also contribute to the exudate.

The exudate observed with pasteurellosis is distinctive in part because of the population of fusiform "streaming" cells[1,8,11,19,20], yet the cause for the "streaming" appearance is unclear. Since necrotic debris and exudate from acetic acid induced lesions had a distinctly different appearance to that associated with pasteurellosis, it seems that the streaming effect is unlikely to be related to mechanical stresses placed on collapsed segments of lungs, since both caused airways obstruction and atelectasis. The cause of "streaming" therefore appears directly related to P. haemolytica. Furthermore "streaming" was not present in calves P7 and P9 indicating that the response occurs only in well developed lesions. Whether this effect is directly related to

some cytotoxic activity of P. haemolytica or due to the effects of liberated inflammatory cell products is unknown. Both mechanisms may act simultaneously.[21-25]

In my research, edema formation often occurred independently and separately from sites of cellular exudate formation and suggests that P. haemolytica may injure the lung by at least two separate mechanisms. Pulmonary edema has been previously suggested as the initial lesion of P. haemolytica pneumonia[2,3], but small numbers of inflammatory cells were also noted in these early lesions. Although the lesions in the lungs tended to have an anteroventral distribution, no differences in the histologic appearance of the lesions were noted suggesting that similar pathogenic processes take place in all lung lobes.

The cause of the vascular damage associated with Pasteurellosis is uncertain. Others[1,11] describe the development of fibrinous thrombi in lymphatics and the flooding of alveoli with proteinaceous fluid that contains fibrillary strands suggestive of fibrin, as was the case in this study. From this study, the distension of lymphatics with fluid and edema of alveolar spaces occurs before thrombosis of lymphatics, indicating that lymphatic thrombosis is not the cause of alveolar edema. Others have reported blood vessel thrombosis as part of pneumonic pasteurellosis[8], but in some studies it was only noted in a proportion of cases[5,12] while in others it was never observed.[11,19,20] One possible cited cause for the vascular injury is endotoxin. Pasteurella sp are potent sources of endotoxin and in calves its administration is associated with pulmonary hypertension, increased pulmonary vascular resistance, and leukocyte migration into the lungs.[26-33] Although species vary in their response to endotoxin, generally endotoxemia is

associated with vascular thrombosis as a result of platelet activation, aggregation and degranulation.[34-36] Therefore, endotoxin may account for some of the vascular damage observed in pasteurellosis of cattle. However, the failure to find vascular thrombosis in my study is incompatible with known actions of endotoxin in cattle.

Functional evidence of ventilation-perfusion ($\dot{V}/\dot{Q}$) inequalities in Pasteurella exposed calves is the increase in alveolar-arterial oxygen difference that occurred in association with decreased dynamic compliance in infected animals. These changes suggest that peripheral airway and parenchymal injury to the lungs leading to hypoventilation of these regions was not accompanied by decreased blood flow to affected regions. Our structural studies support this conclusion because many lobules were atelectatic, with exudate obstructing the bronchioles and yet had pronounced vascular engorgement of alveolar capillaries. Endotoxin might disturb $\dot{V}/\dot{Q}$ matching because of its potent effects on normal bovine pulmonary vasculature.[32] Other factors are likely to be involved; gram positive infections of other species cause similar $\dot{V}/\dot{Q}$ mismatching in the lungs[37] but gram positive organisms do not synthesize endotoxin. The source for such vascular changes many come from bacterial products other than endotoxin, such as cytotoxins[21,22] and bacterial kallikreins.[38]

Other sources for the vascular and tissue injury observed in pulmonary pasteurellosis are the cellular products of platelets and leukocytes. The release of leukocyte lysozomal products, platelet biogenic amines, prostaglandins and leukotrienes synthesized by macrophages, kallikreins and proteases can cause tissue damage in the lungs of other species and may contribute to the severity of pasteurella induced

injury.[23-25,34] The development of irregularly shaped areas of coagulative necrosis in lungs affected with pasteurellosis for 18 hrs or more has been thought the result of vascular thrombosis and infarction.[1,8,10,11] The lesions we observed in calves P6 and P8 developed without widely distributed vascular thrombosis but were always associated with an intense infiltrate of macrophages and lesser numbers of other inflammatory cell types. Tissue necrosis may therefore be associated in some way with these inflammatory cells. It is uncertain whether these necrotic areas only occur in developed lesions because of a necessity to accummulate large numbers of inflammatory cells, or whether the response is related to bacterial products which are necrotizing and which reach toxic levels when bacterial numbers increase sufficiently following inoculation.

In physiologic studies of pneumonic calves, I was unable to detect alterations in functional residual capacity (FRC) despite atelectasis and hepatization of diseased portions of lung. I concluded that remaining ventilated portions of lung would become hyperinflated in order for FRC to remain constant. Structural studies demonstrated that areas of hyperinflation and emphysema did occur, these being described in some but not all descriptions of P. haemolytica pneumonia summarized by Rehmtulla and Thompson.[1] Emphysematous changes were only detected on histologic evaluations of pneumonic lungs in my study but grossly visible emphysematous bullae have also been described in calves infected with P. haemolytica.[39]

In a companion paper, I discuss theoretical reasons why plasma histamine levels may not adequately reflect tissue histamine levels if pasteurellosis was to cause mast cell degranulation. Preliminary

surveys of Toluidine Blue stained plastic sections did not reveal marked differences between populations of mast cells associated with the major airways of control and Pasteurella exposed calves, further indicating that histamine is unlikely to be an important mediator of pulmonary injury associated with pasteurellosis.

Control calves and Pasteurella exposed calves had lesions associated with the small intestine and liver. The liver lesions may have developed as a result of portal venous or biliary transportation of hepatotoxic substances or bacteria from the inflamed intestine. It is not known whether cold stress facilitated the lesions. The finding of mild perivascular cuffing of the brain in 2 calves infected with pasteurellosis, thrombosis of a small arteriole in the cerebrum of P3 and one calf with a microabscess also in the brain, may have been unrelated to the Pasteurella lesions. No bacteria were observed in specially stained histologic slides of affected regions, but thrombosis of neighboring vessels may account for these lesions. The possibility of a circulating factor released from the lungs of calves with pasteurellosis cannot be excluded, but would seem unlikely since only 2 of 9 calves had the lesion and both calves with brain lesions had relatively mild lung disease. Both groups of calves had some evidence of extramedullary hematopoiesis and had narrow zones of small lymphocytes occupying the splenic white pulp. These findings have previously been described in normal neonatal calves[40] and therefore were not the results of stress. The same authors describe typical lymph nodes of neonatal calves, which were similar to control calves in this study.

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CHAPTER 5

CONCLUSIONS

CONCLUSIONS

Epidemiological studies have clearly incriminated Pasteurella haemolytica as a prime factor in the injury of the lungs associated with Shipping Fever, and have also identified the importance of stress, particularly thermal stress and that resulting from transportation, in predisposing to pneumonia. The mechanisms that act to cause the initial lung injury in cattle with Shipping Fever are unknown. This study was designed to describe the development of initial pulmonary injury by structural and functional means and to test several hypotheses regarding postulated mechanisms of injury.

I. Cold Stress

Cold stress and spraying of the trachea with acetic acid resulted in a focal necrotizing tracheitis and isolated lobules of lung had hemorrhage, edema and often cellular infiltrates accumulating in the alveolar lumens when examined 12 hrs after the cold stress. These lesions did not cause any functional changes in the lungs, and may have arisen because of acetic acid inhalation into the lungs or because of early bacterial pneumonia. From the data obtained in this study, it was not possible to determine whether the mixed bacterial types cultured from control calf lungs were the cause of the focal pneumonic areas or were simply isolated from the lungs without exerting pathogenic effects. Cold stressed calves had no change in their hemograms immediately following stress but plasma total solids, erythrocyte numbers, hematocrit and hemoglobin content decreased over the remainder of the experiment presumably because of repeated sampling of blood. The leukogram was maintained at baseline levels in the control calves,

indicating the addition of leukocytes to the circulating blood occurred in order to replace losses through repetitive blood sampling. There was no change in plasma bradykinin and histamine levels, serum thyroxine or triiodothyronine but serum cortisol was significantly increased by cold stress. These data indicate that thyroid hormones, mast cell products and humoral factors which may activate the kinin system (principally the intrinsic clotting system) are not involved in responses to cold stress. The importance of cold-stress induced corticoid release is unclear since it did not affect circulating leukocyte numbers. These experiments did not determine leukocyte function so that the specific effects of cold stress on corticoid-induced immunosuppression were not determined.

Cold stress increased alveolar ventilation because tidal volume was increased while minute ventilation was maintained at baseline levels. The increased alveolar ventilation was necessitated by an increased metabolic demand for oxygen uptake and CO_2 excretion from the lungs so that no change in arterial O_2 or CO_2 tension occurred. While such respiratory adjustments function well to preserve homeostasis in the face of thermal stresses, they may increase the load of inspired pathogens delivered into the respiratory exchange area. Since P. haemolytica is normally present in aerosolized droplets generated from the nasal cavity during normal breathing, increased alveolar ventilation may be an important mechanism for predisposing to Shipping Fever during changes in body metabolism induced by thermal stress.

II. Effects of Pasteurella haemolytica

The response of calves to P. haemolytica exposure seemed to involve three phases. An initial phase, measurable within one hour of challenge and extending for about 3 hours, resulted in alterations in the pattern of breathing and in gas exchange function but was not accompanied by changes in the hemogram or leukogram. The onset of injury at this time was associated with maintenance of serum cortisol levels above baseline. Alterations in the respiratory rate resulted in increased dead space ventilation but dead space ventilation increased above the level predicted by changes in frequency alone. In addition, arterial oxygen decreased despite a maintenance of alveolar ventilation. These changes indicate the development of ventilation-perfusion inequalities within the lungs. Since the impairment of gas exchange did not worsen from 3 to 6 hours post inoculation but respiratory rate returned to baseline levels, these data indicate that the initial stimulus for increased respiratory rates was not hypoxemia and probably was reflex in origin. Because irritant receptor stimulation leads to reflex bronchoconstriction, and since no functional evidence of bronchoconstriction was present, it is probable that reflex stimulation of respiration arose through intrapulmonary J receptor stimulation. These receptors are stimulated by developing pulmonary edema, which was a consistent histologic lesion present in all calf lungs exposed to P. haemolytica.

The second phase was ushered in from three to less than 12 hrs post inoculation by decreased dynamic compliance and increased alveolar-arterial oxygen difference. The pattern of breathing had returned to normal but there was an increase in dead space ventilation. These data indicate continued gas exchange impairment and peripheral airway and

lung parenchymal injury caused changes in compliance. The histologic findings of calves dying before 12 hrs supports these conclusions. Furthermore, the concentration of pneumonic lesions in the anterior lobes probably resulted in a redistribution of ventilation to the diaphragmatic lobes. This redistribution further increases dead space and since it did not appear to be accompanied by a redistribution of perfusion away from pneumonic tissues, based on the histologic appearance of pneumonic lung, probably led to exacerbation of ventilation-perfusion mismatching.

The third phase of the disease had occurred by 12 hours post inoculation, and was characterized by hypoventilation and increased airway resistance in addition to changes previously described. These changes indicate that acute respiratory failure had developed by this time and that the extensive alterations in the pulmonary mechanical properties probably resulted in respiratory muscle failure. Because functional residual capacity (FRC) did not decrease in calves with pneumonia, hyperinflation of the remaining healthy portions of lung occurred. Since these portions of lung then operate at greater inflations than normal, the failure of calves to reduce FRC during the development of pneumonia further exacerbated the decrease in dynamic compliance and probably hastened respiratory failure.

Data from the study of hematologic and hormonal variables during this third phase indicated that P. haemolytica pneumonia resulted in neutropenia and increased serum cortisol. Histologic studies support the importance of the neutrophil in the initial response to Pasteurella haemolytica injury of the lungs. It is unknown whether neutrophils and the other inflammatory cells of the lungs contribute to pulmonary injury

in this disease. Histologically, the processes of pulmonary edema and inflammatory cell infiltration into the lungs appeared to occur independently suggesting that alveolar wall injury can occur independently from leukocyte action. The large irregular zones of necrosis that begin to develop in calf lungs exposed to P. haemolytica for 12 hrs were always associated with cellular exudates and may indicate injury mediated by inflammatory cell products at this time.

There was no evidence to support a role of histamine in the pathogenesis of pneumonic Pasteurellosis. Histamine was not released into the blood, nor were there physiologic or histologic changes compatible with the generation of histamine. Similarly, the data do not support a role for bradykinin. This was surprising since several possible sources of kinin pathway activation seemed to be present. The principle stimulus for kinin generation is activated Hageman factor, and such activation was thought likely because of endotoxin and should result in vascular thrombosis. Vascular thrombosis was not a feature of this disease, although histologic methods to detect intra-alveolar capillary thrombosis may have been insensitive. These data therefore suggest that thrombosis and kinin production are not a feature of the disease and that if endotoxin is important in the pathogenesis of pneumonic Pasteurellosis, it has no effect on the bovine kinin system.

APPENDIX A

The following variables were measured for the determination of pulmonary gas exchange:

Expired O_2 partial pressure	P_EO_2
Expired CO_2 partial pressure	P_ECO_2
Expired N_2 partial pressure	P_EN_2
Inspired N_2 partial pressure	P_IN_2
Inspired O_2 partial pressure	P_IO_2
Tidal volume	V_T
Inspired oxygen fraction	$F_{I_{O_2}}$
Expired oxygen fraction	$F_{E_{O_2}}$
Inspired CO_2 fraction	$F_{I_{CO_2}}$
Expired CO_2 fraction	$F_{E_{CO_2}}$
Barometric pressure	P_B
Arterial oxygen tension	PaO_2
Arterial CO_2 tension	$PaCO_2$
Respiratory frequency	f

From these measurements the following formulae were used in order to calculate the aspects of gas exchange outlined below.

1. Alveolar oxygen tension (PAO_2)

$$PAO_2 = F_{I_{O_2}} (P_B - P_{H_2O}) - \frac{PACO_2}{R_E} + \left[F_{I_{O_2}} \times P_ACO_2 \times \frac{(1-R_E)}{R_E}\right]$$

where P_{H_2O} is the water vapor pressure at body temperature and where

$$R_E = \frac{P_ECO_2 \times P_IN_2}{P_IO_2 \times P_EN_2} - P_EO_2$$

The equation is solved by assuming that $PACO_2$ is closely approximately by $PaCO_2$.

2. Alveolar-arterial oxygen difference ($AaDO_2$)

$$AaDO_2 = PAO_2 - PaO_2$$

3. Dead space/tidal volume ratio (V_D/V_T)

$$V_D/V_T = \frac{P_ACO_2 - P_ECO_2O}{P_ACO_2}$$

This equation is solved by substituting $PaCO_2$ in place of $PACO_2$.

4. Alveolar ventilation ($\dot{V}_A$)

$$\dot{V}_A = \dot{V}_{MIN}(1-V_D/V_T)$$

where $\dot{V}_{MIN} = V_T \times f$

5. Dead space ventilation ($\dot{V}_D$)

$$\dot{V}_D = \dot{V}_{MIN} - \dot{V}_A$$

6. CO_2 production ($\dot{V}_{CO_2}$)

$$\dot{V}_{CO_2} = \dot{V}_{MIN} \frac{[F_ECO_2(1-F_IO_2)-F_ICO_2(1-F_EO_2)]}{1-F_{I_{O_2}}-F_{I_{CO_2}}}$$

Assuming that $F_ICO_2 = 0$ then the equation simplifies to $\dot{V}_{CO_2} = \dot{V}_{MIN} \times F_ECO_2$.

7. Oxygen consumption ($\dot{V}_{O_2}$)

$$\dot{V}_{O_2} = \dot{V}_{MIN} (F_IO_2 - F_EO_2)$$

APPENDIX B

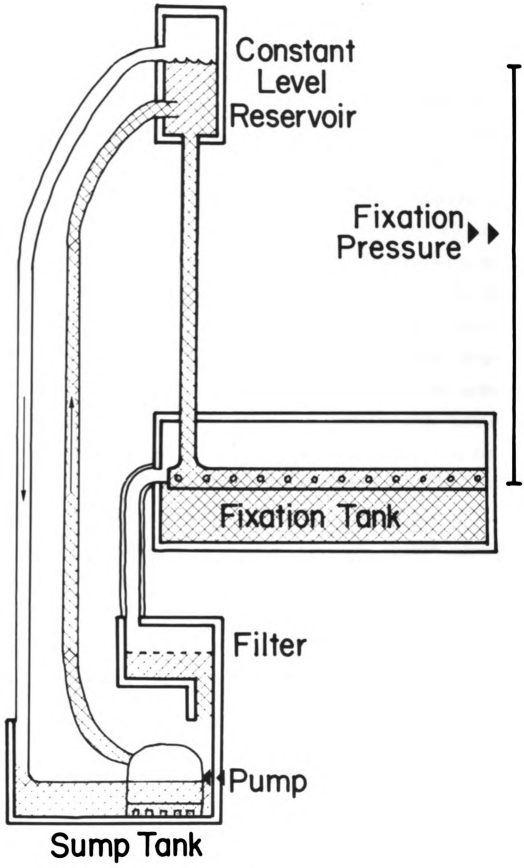
The apparatus used for fixation of lung specimens is illustrated (see Figure). A fixation tank, constructed of plexiglass, was made with a centrally positioned manifold containing exit ports for passage of pressurized fixative. Exit ports were made of standard stainless steel catheter connections. Samples of lung tissue, with major bronchi catheterized and tied firmly in place, were connected to the exit ports of the fixation tank manifold. The samples were submerged under fixative in the fixation tank and were also fixed by pressure perfusion of the airways via the bronchial catheter. Effluent fixative escaping from the lungs drained from the fixative tank into a sump after passing through a filter.

Fixative was delivered to a pressure reservoir from the sump by a continuously running, magnetically driven, corrosion resistant submersible pump. The height of fixative in the pressure reservoir was maintained constant by way of a continuous overflow into a wide base sump return pipe. Fixative entered the manifold in the fixation tank by a connecting pipe from the base of the pressure reservoir, and the pressure at the level of the exit ports in the manifold altered by raising or lowering the pressure reservoir in relation to the fixation tank.

By this means lung tissue was fixed by a combination of airway perfusion under constant hydrostatic pressure head and also by submersion.

Appendix B, Figure 1

Schematic diagram of the apparatus used in the fixation of lung samples.



Appendix B

Figure 1

VITA

The author was born at Bacchus Marsh, Victoria, Australia, in 1951. He attended small country schools for his primary and secondary school education, matriculating first in his class at Ballarat East High School in 1969. He enrolled in the veterinary curriculum at the University of Melbourne, Melbourne, Australia, in 1970, graduating with first class honors in 1974. After 18 months as an assistant in mixed veterinary practice in central Alberta, Canada, the author joined the faculty at Michigan State University (MSU) in July, 1976, for pursuit of a residency in Food Animal Medicine and also concurrently enrolled in a Masters Degree program with the Department of Large Animal Surgery and Medicine. After completion of these programs in 1979, the author joined the Department of Large Animal Surgery and Medicine as an instructor and the Pathology Department as a graduate student enrolled in a PhD program. The author completed his PhD in Pathology in 1982. During his appointments at MSU, the author was nominated to Phi Zeta, Phi Kappa Phi and Sigma Xi, and was the recipient of a graduate student award in Sigma Xi for his research achievements.