SERUM XANTHINE OXIDASE ACTIVITY IN DOGS WITH ISCHAEMIC DISORDERS

A thesis presented in partial fulfilment of the requirements for the degree of Master of Veterinary Science at Massey University

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1997

Abstract

This thesis has focused on the measurement of the serum xanthine oxidase activity in dogs with diseases which involve ischaemia-reperfusion injury. The pathophysiology of ischaemia-reperfusion injury, the production of oxygen derived free radicals (ODFRS), their deleterious effects, the endogenous protective mechanisms against ODFRS, and the structure, function, distribution and kinetics of xanthine oxidase, have been reviewed.

Xanthine oxidase activity in blood and tissues can be measured using a variety of assays of uric acid production over time. A spectrophotometric assay was developed for use with canine serum, and studies were undertaken to assess the linearity and reproducibility of the assay. The effect of storage temperature and duration on the activity of bovine milk xanthine oxidase in canine serum, was investigated. The serum xanthine oxidase activity was measured in "healthy" dogs, and in dogs presented to the veterinary clinic with diseases likely to involve ischaemia-reperfusion injury.

Xanthine oxidase activity followed zero order kinetics after a short burst phase. The intra-assay and inter-assay coefficients of variation were less than or equal to 5.5% and 12.8%, respectively. Bovine milk xanthine oxidase was stable in serum stored at -20°C or -80°C for 90 days. A wide range of serum xanthine oxidase activity were measured in clinically "healthy" dogs (0-363 mU/l) and values obtained did not assume a Gaussian distribution. Using nonparametric methods, a reference interval, containing 95% of the xanthine oxidase activities, was determined to be 0-204 mU/l. The serum xanthine oxidase activity was not dependent upon age or sex.

Compared with "healthy" dogs, the sick dogs had significantly higher serum xanthine oxidase activities. The serum xanthine oxidase activity was significantly higher following reperfusion (treatment with intravenous fluids), than prior to treatment. There was a statistically insignificant trend towards higher serum xanthine oxidase activities in dogs with more severe clinical signs relating to the cardiovascular system, but the serum xanthine oxidase activity did

not appear to be useful in predicting patient survival.

Circulating xanthine oxidase may be involved in the development of complications that are seen relatively frequently following ischaemia-reperfusion injury in dogs. Xanthine oxidase may react with purine substrates in the plasma, producing large amounts of ODFRS throughout the body, resulting in widespread capillary endothelial damage, and the attraction of inflammatory cells into organs some distance from the original site of ischaemia and reperfusion.

Acknowledgements

Funding was provided by the Massey University Veterinary Research Fund, and the Massey University Research Fund. I would like to thank the clients, students and staff members who allowed the collection of blood from their dogs, and the nursing staff, students and my colleagues for their assistance in blood collection, and care of the dogs during hospitalisation. I would also like to thank Mark Wiseman for his input into the development of the spectrophotometric assay, and Steve Haslett, who performed the statistical analysis in Chapters 3 and 4.

I am extremely grateful for the support, encouragement and wisdom provided by Grant Guilford, Boyd Jones and Hilary Burbidge during my residency. Thank you for sharing your enthusiasm for veterinary medicine, and for providing the inspiration and opportunity to learn. In particular, I thank Grant for the time and advice he has generously provided during the preparation of this thesis.

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Chapter 1

The Role of Xanthine Oxidase in Ischaemia-Reperfusion Injury

Introduction

Ischaemia-reperfusion injury is the injury that occurs when the blood supply is restored to previously ischaemic tissues (Badylak 1990). It is a common cause of morbidity and mortality in dogs (Guilford 1990). Xanthine oxidase is an enzyme that is intimately involved in the pathophysiology of ischaemia-reperfusion injury (Granger *et al* 1981, Roy and McCord 1983, Sarnesto *et al* 1996).

Ischaemia-reperfusion injury affects most body tissues including the gastrointestinal, respiratory and urinary tracts, and the central nervous system (Beckman *et al* 1986, Toledo-Pereyra and Suzuki 1994). Gastric dilatation-volvulus is an example of ischaemia-reperfusion injury. Distention and rotation of the stomach results in occlusion of the blood supply, and ischaemia of the stomach. Reperfusion occurs when the stomach is decompressed and returned to its correct position, and intravenous fluids are administered to increase circulating blood volume (Guilford 1990). Ischaemia reperfusion injury may also play a role in the pathophysiology of gastrointestinal diseases such as parvoviral enteritis, haemorrhagic gastroenteritis, gastrointestinal obstruction and pancreatitis, and in clinical problems such as heatstroke, pyometra, shock (hypovolaemic, endotoxic, septic) and immune mediated haemolytic anaemia (see section 1.4).

While the resupply of oxygen is essential for restoration of normal cell function, its return to previously ischaemic tissues initiates a chain of events, often with catastrophic effects (Granger and Korthuis 1995). Reperfusion of ischaemic tissue results in microvascular injury (Menger 1995), ultrastructural cellular changes, and may ultimately lead to cell death (Rochat 1991). Some cell and tissue damage occurs during the ischaemic phase (Chiu *et al* 1970), and the extent and reversibility of such injury is dependent upon the duration and severity of the ischaemia

(Brown *et al* 1970, Snyder 1989, Granger and Korthuis 1995). However, reperfusion results in a series of biochemical reactions, which exacerbate the damage produced by ischaemia alone (Parks and Granger 1986b). This has been demonstrated in a number of studies. For example, Parks and Granger (1986b) showed that 4 hours of intestinal ischaemia resulted in less tissue damage than 3 hours of ischaemia followed by 1 hour of reperfusion. Using an experimental model of canine gastric dilatation-volvulus, Guilford (1990) showed that minimal histopathological changes were found in the intestine and liver after 2 and a half hours of ischaemia, but changes were marked after 4 hours of reperfusion. Also, Hearse *et al* (1973) found that the release of creatine kinase (an indicator of myocardial cell membrane injury) from *in vitro* ischaemic rat hearts did not begin until reperfusion, and Perry *et al* (1988) found that injury to the gastric mucosa of cats was minimal during the ischaemic phase (<10% of the total injury), and marked with reperfusion. Parks and Granger (1986b) also showed that reperfusion with deoxygenated blood for one hour following 3 hours ischaemia, was no more damaging than 3 hours of ischaemia alone, implying that it is the reintroduction of oxygen which initiates the damaging sequence of events.

1.1 Pathophysiology of Ischaemia-Reperfusion Injury

The pathophysiology of ischaemia-reperfusion injury can be divided into three phases:

1. The conversion of the enzyme xanthine dehydrogenase to xanthine oxidase, and the accumulation of substrate and inflammatory cells within ischaemic tissues
2. The restoration of circulation (return of oxygen) and subsequent generation of oxygen derived free radicals (ODFRs), and further inflammatory cell infiltration
3. The initiation and propagation of membrane lipid peroxidation and cell protein oxidation by oxygen derived free radicals (Badylak *et al* 1987).

In addition the no reflow phenomenon may play a role in the resultant tissue injury in some situations (Menger 1995).

(a) Phase One - Conversion of Xanthine Dehydrogenase to Xanthine Oxidase, Neutrophil and Substrate Accumulation

Under normal aerobic conditions, cells utilise oxygen, glucose and fatty acids, to generate the major cellular energy source, adenosine triphosphate (ATP), using the mitochondrial enzyme system, cytochrome oxidase (Winrow *et al* 1993). Under hypoxic conditions (the ischaemic phase), ATP is depleted (Lindsay *et al* 1990, Conger and Weil 1995) and adenylate (adenosine monophosphate or AMP) accumulates within cells (Grisham and Granger 1989). Adenosine triphosphate depletion is complete within 20 minutes of total ischaemia in the rat small intestine (Blum *et al* 1986). Adenylate is converted to hypoxanthine through the intermediate metabolic products adenosine and inosine (Hearse *et al* 1986, Hammamoto *et al* 1993). A 10 to 20 fold elevation in hypoxanthine has been measured in rat and cat intestine during the ischaemic phase (Mousson *et al* 1983, Schoenberg *et al* 1985). Hypoxanthine is unable to be further metabolised under hypoxic conditions, but is the substrate on which xanthine oxidase will work once oxygen is resupplied to the tissue (Rochat 1991).

The Na^+K^+ATPase pump is a transmembrane protein which maintains the normal ionic gradient across the plasma membrane (Huang *et al* 1992). Without ATP the pump fails, resulting in a loss of cell membrane integrity, and sodium enters the cell (Flitter 1993). Calcium enters the cell in exchange for intracellular sodium, resulting in the intracellular accumulation of calcium, which activates proteases (including calpain) and phospholipases (Opie 1989). Calpain converts the enzyme xanthine dehydrogenase to xanthine oxidase (McCord 1985) (see section 1.3 for more details). Proteases and phospholipases also damage cell membranes, leading to the breakdown of arachidonic acid via the cyclooxygenase and lipoxygenase pathways, to form prostaglandins and leukotrienes. Some oxygen derived free radicals are produced as byproducts of these pathways (Hitt 1988) (see section 1.2 part b).

Neutrophils are attracted to damaged, hypoxic tissue by chemotactic factors such as platelet activating factor, leukotrienes, complement 3a and 5a, bacterial substances (Moore *et al* 1994, Granger and Korthuis 1995) and physical disruption *per se* during the ischaemic period (Flitter

1993). A five to seven fold increase in neutrophil numbers occurs within one hour of partial ischaemia of the feline intestine (Grisham *et al* 1986). Neutrophil infiltration may not occur during complete ischaemia (Anderson *et al* 1994). Oxygen derived free radicals do not appear to play a role in neutrophil chemotaxis during this phase, as pretreatment with xanthine oxidase inhibitors and ODFR scavengers have not prevented neutrophil influx in experimental feline intestinal ischaemia-reperfusion models (Grisham *et al* 1986, Zimmerman *et al* 1990).

(b) Phase two - Restoration of Circulation, the Generation of Oxygen Derived Free Radicals and Further Influx of Inflammatory Cells

The resupply of oxygen to previously ischaemic tissues results in the production of large amounts of oxygen derived free radicals (ODFRs) (Nilsson *et al* 1987, Morris *et al* 1987). The main sources are the enzyme xanthine oxidase, and the phagocytic cell enzymes, NADPH oxidase, NADH oxidase and myeloperoxidase (Moore *et al* 1994).

When oxygen is present, xanthine oxidase catalyses the conversion of hypoxanthine to xanthine, and then to uric acid (Rochat 1991), forming superoxide radical and hydrogen peroxide as byproducts (see Figure 1.8). Superoxide radical can also spontaneously, or enzymatically dismutate to hydrogen peroxide (Weiss 1986). Superoxide and hydrogen peroxide can combine to form the hydroxyl radical via the Haber Weiss Reaction (see Figure 1.1). This reaction is too slow to be of biological significance, but in the presence of a transition metal catalyst, such as iron, hydroxyl is formed through Fenton Chemistry (Kirschner and Fantini 1994) (see Figure 1.2). Ferritin and mitochondrial cytochromes are sources of iron for the Fenton reaction (Granger 1988) and the hydrogen ions required for the reaction are plentiful in ischaemic tissue, due to accumulation of lactic acid and other acids following anaerobic metabolism (Rochat 1991).

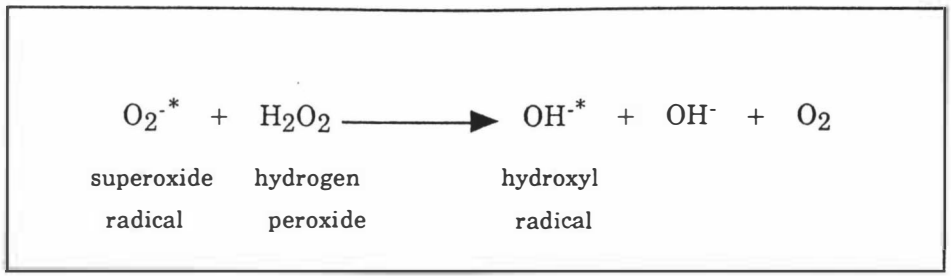


Figure 1.1 The Haber Weiss Reaction

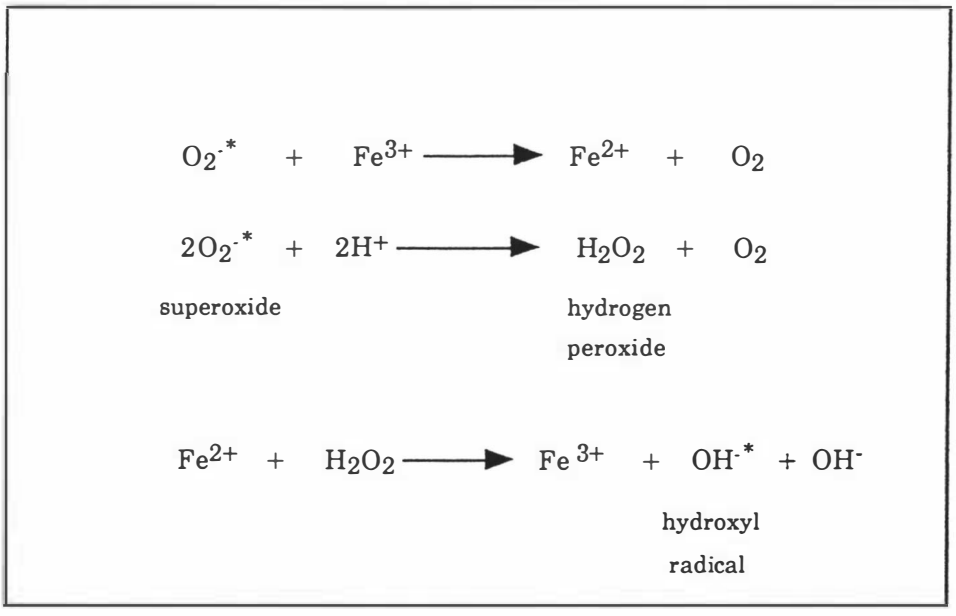


Figure 1.2 The Formation of Hydroxyl Radical by Fenton Chemistry

(From Kirschner and Fantini 1994)

The phagocytic cell enzymes, NADPH oxidase and myeloperoxidase, are the other important source of ODFRs in ischaemia-reperfusion injury. Neutrophil infiltration, which begins during the ischaemic period, is greatly enhanced during reperfusion. An 18 fold increase in neutrophil numbers occured within 1 hour of reperfusion following 3 hours of ischaemia of the feline small intestine (Grisham *et al* 1986).

Neutrophils are attracted to the site of tissue injury by chemotactant substances. Xanthine oxidase derived ODFRs appear to play a key role in the chemotaxis of neutrophils, and adhesion to the vascular endothelium during the reperfusion phase (Kubes and Granger 1992) (see section 1.2 for details). This is supported by the finding that neutrophil influx during the reperfusion phase can be attenuated by pretreatment with free radical scavengers and allopurinol (xanthine oxidase inhibitor) in experimental intestinal ischaemia- reperfusion injury (Grisham *et al* 1986, Zimmerman *et al* 1990, Kubes and Granger 1992). Other chemotactants such as complement 3a and 5a, and leukotriene B_4 are also likely to be involved (Moore et al 1994).

Phagocytes (neutrophils, monocytes, macrophages) provide host defence by binding, ingesting and destroying pathogenic microorganisms, by the production of ODFRs and toxic lysosomal enzymes (Weiss 1986, Patel *et al* 1991). These products are also responsible for some of the tissue damage in ischaemia-reperfusion injury. The NADPH oxidase system is an electron transport chain situated in the cell membrane of phagocytic cells (Moore *et al* 1994). "Resting" phagocytes have very low, or no NADPH oxidase activity (Forman and Thomas 1986), but become stimulated following cell membrane contact with antigen (bacteria, viruses and bacterial products) or immune complexes (Hitt 1988), and undergo a short period of increased oxygen consumption known as the respiratory burst (VanSteenhouse 1987). During this burst, NADPH oxidase and the hexose monophosphate shunt are activated. Activation of the latter leads to the generation of large amounts of NADPH (Winrow *et al* 1993) which acts as an electron donor to oxygen, producing superoxide radical (Hitt 1990) (see Figure 1.3). Superoxide is released into the vacuole that forms when the phagocytic cell membrane engulfs the antigen or antibody-antigen complex (Hitt 1988). The release of superoxide is essential for bacterial killing by neutrophils (Forman and Thomas 1986). Superoxide is also released outside the cell, to act directly or indirectly, as a chemotactant (Hitt 1988).

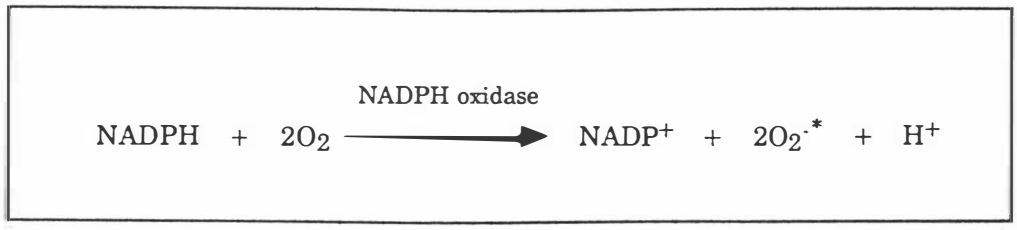


Figure 1.3 The Formation of Superoxide Radical by NADPH oxidase

The phagocytic vacuole also fuses with lysosomes containing the enzyme myeloperoxidase (Flitter 1993). This enzyme catalyses the oxidation of halides by hydrogen peroxide, to form hypohalous acids (Weiss 1986) (see Figure 1.4). The predominant hypohalous acid produced is hypochlorous acid, formed by the oxidation of chlorine. Other hypohalous acids formed include hypobromous, hypoiodous and hypothiocyanous acid (Weiss 1986). Hypochlorous acid is a powerful oxidant (Weiss 1986), and can react with amines to form N-chloramines. Both substances are bactericidal (Forman and Thomas 1986).

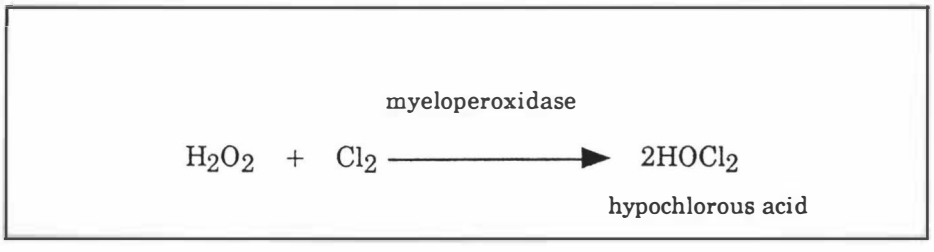


Figure 1.4 The Production of Hypochlorous Acid by Myeloperoxidase

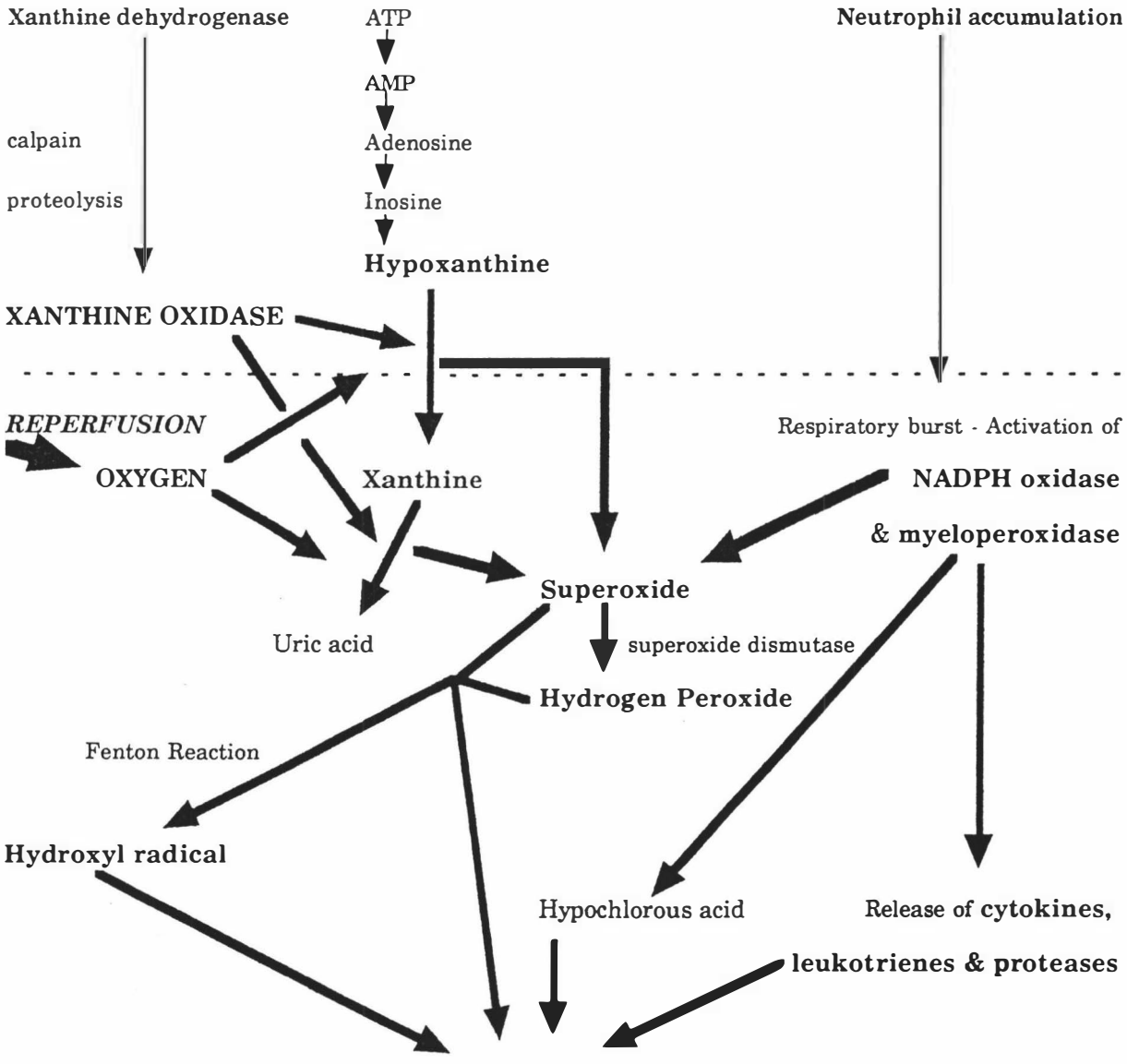
Neutrophils also produce proteases (such as elastase), collagenase and cationic proteins which damage tissue (Snyder 1989, Hernandez 1987), and cytokines and leukotrienes which provide further chemotaxis to increase the severity of the inflammation (Snyder 1989). Proteases can also inactivate enzyme antioxidants such as superoxide dismutase and catalase, reducing host defences (Rangan and Bulkley 1993).

(c) Phase three - Initiation and Propagation of Lipid Membrane Peroxidation and Cellular Protein Oxidation by Oxygen Derived Free Radicals

Radicals can peroxidise lipid membranes by extracting a hydrogen ion from a polyunsaturated fatty acid side chain, forming an alkyl radical (Halliwell 1994). A chain reaction of lipid peroxidation is initiated. Oxygen derived free radicals can damage membrane proteins (receptors, enzymes and transduction systems), intercellular binding proteins (fibronectin), hyaluronic acid, collagen and deoxyribonucleic acid (DNA) (Hitt 1990). Further information on the destructive effects of ODFRs is given in section 1.2. The end result of ischaemia-reperfusion injury is endothelial and parenchymal cell injury, increased capillary permeability, interstitial oedema, inflammation and organ dysfunction (Zimmerman *et al* 1990, Menger 1995).

The pathophysiology of ischaemia-reperfusion injury is summarised in Figure 1.5. Oxygen derived free radicals derived from xanthine oxidase play an important role via direct tissue injury and chemoattraction of inflammatory cells. Evidence for their involvement in ischaemia-reperfusion injury is largely derived from studies showing a protective effect of pretreatment with inhibitors of xanthine oxidase, such as allopurinol (Adkison *et al* 1986, Granger 1988, Mink *et al* 1991, Asami *et al* 1996), or protease inhibitors, which prevent the conversion of xanthine dehydrogenase to the oxidase form (Parks and Granger 1983, Parks *et al* 1985). In addition, exposure of tissues to xanthine oxidase and xanthine results in similar tissue injury to that seen in ischaemia-reperfusion injury (Parks *et al* 1984). Moreover, oxygen derived free radical formation has been identified in reperfused ischaemic tissues, using electron spin resonance spectroscopy and electron spin trapping (Mason and Morehouse 1989), and evidence of lipid peroxidation is present in reperfused tissues (Granger and Korthuis 1995).

ISCHAEMIA



Tissue Damage (lipid peroxidation, protein oxidation etc)

Inflammatory Cell Chemotaxis

Inflammation

Cell Death

Organ Failure

Figure 1.5 The Pathophysiology of Ischaemia-Reperfusion Injury

(d) The"No Reflow" Phenomenon

If blood flow is not re-established to ischaemic tissues, then ongoing damage will obviously occur. This is called the "no reflow" phenomenon (Menger 1995, Forsyth and Guilford 1995). Without oxygen, the cells' energy source, adenosine triphosphate, is depleted and is unable to be regenerated, cellular ion pumps fail and cell swelling and death occur (Rochat 1991). Persistent vasoconstriction, vascular obstruction (as a result of thrombus formation, neutrophil margination or interstitial oedema), hypotension, hyperviscosity (due to plasma leakage across capillary endothelial cells) and release of vasoactive agents like platelet activating factor and norepinephrine, may prevent the adequate reflow of blood (Snyder 1989, Conger and Weil 1995, Menger 1995).

A venous obstruction is more likely to result in the no reflow phenomenon than an arterial obstruction for several reasons. Venous obstruction results in increased capillary hydrostatic pressure, tissue oedema and consequently elevated perivascular pressure, compressing the microcirculation. Thrombus formation is also more likely in a venous obstruction due to vascular pooling (Guilford 1990). In addition, the no reflow phenomenon is more likely to occur with long periods of ischaemia (Menger 1995).

Xanthine oxidase and ODFRs may play a role in the "no reflow" phenomenon, as Punch et al (1992), showed that pretreatment with allopurinol or free radical scavengers prevented the occurrence of "no reflow", induced by prolonged ischaemia of the rat hindlimb.

(e) The Relative Importance of Xanthine Oxidase Derived Oxyradicals and Granulocytes in Ischaemia Reperfusion Injury

Free radical mediated ischaemia-reperfusion injury appears to begin at the microvascular level, with ODFRs directly damaging the vascular endothelium, and promoting the attraction of neutrophils, their adhesion to vascular endothelial cells and subsequent tissue infiltration (Rangan and Bulkley 1993). Oxygen derived free radicals are not the sole mediators of ischaemia-reperfusion injury and inflammation. Lymphokines, acute phase proteins, complement, prostaglandins, leukotrienes are also involved (Hitt 1988).

Neutrophils are generally considered to be the primary mediators of the tissue injury, with xanthine oxidase derived ODFRs making a major contribution to the initiation of the inflammatory response (Kubes and Granger 1992). Experimental depletion of neutrophils, or prevention of neutrophil adherence and extravasation in *in vivo* models of gastrointestinal ischaemia-reperfusion injury, by pretreatment with antineutrophil serum and monoclonal antibodies, has resulted in attenuation of the tissue damage (Grisham *et al* 1987, Andrews *et al* 1994) and the increase in microvascular permeability associated with reperfusion (Hernandez *et al* 1987, Carden *et al* 1990). Andrews *et al* (1994) estimated that approximately half of the tissue injury associated with gastric ischaemia-reperfusion injury in rats, was due to xanthine oxidase derived ODFRs, with the remaining injury due to neutrophil infiltration and subsequent production of more ODFRs, proteases and inflammatory mediators.

1.2 Oxygen derived free radicals

(a) Classification of radicals

A radical is a molecule with an unpaired electron in its outer orbit (Kirschner and Fantini 1994). Radicals are formed by the addition or loss of an electron from a "regular" molecule, or less frequently, cleavage of a covalent bond in a molecule to form two radicals, each with one unpaired electron (Cheeseman and Slater 1993). "Regular" molecules have equal numbers of electrons spinning in opposite directions, making the molecule relatively stable (VanSteenhouse 1987).

Oxygen derived free radicals (ODFRs), also known as oxyradicals, are derived from molecular oxygen. Oxygen is an unusual molecule in that it has an unpaired electron in two orbitals, so it can lose or gain electrons easily (Hitt 1988). The oxygen derived free radicals include superoxide, hydroxyl, peroxyl and alkoxyl radicals (Halliwell 1994), nitrogen dioxide, hydroperoxyl, acyloxyl, acylperoxyl, aryloxyl and arylperoxyl radicals (Pryor 1986). Hydrogen peroxide is not a true radical, but is included in the group of reactive oxygen species (Cheeseman and Slater 1993) as it can be broken down to form the hydroxyl radical (Badylak 1990).

(b) Production of oxygen derived free radicals

Oxygen derived free radical production can be accidental or deliberate, generated as byproducts of normal cellular metabolism or subsequent to pathological stimuli (Hearse *et al* 1986).
Sources of oxygen derived free radicals are:

1. Xanthine oxidase (McCord 1985)
2. Phagocytic cell enzymes, NADPH oxidase and myeloperoxidase (Kubes and Granger 1992)
3. Leakage of electrons from electron transport chains - cytochrome oxidase (Grisham and Granger 1989), microsomal mixed function oxidase (Cytochrome p450) (Halliwell and Gutteridge

1985), endoplasmic reticulum and nuclear membrane electron transport systems (Weiss 1986)

4. Catecholamine degradation by monoamine oxidase (Hearse *et al* 1986)

5. Cyclo-oxygenase and lipoxygenase pathways in arachidonic acid metabolism (Hitt 1988))

6. Exogenous sources - ionising radiation and drugs (doxorubricin)

7. Endogenous auto-oxidisable compounds eg thiols like glutathione and cystein, flavins and coenzymes (Cheeseman and Slater 1993)

8. Self generation (Weiss 1986)

Xanthine oxidase is the major source of superoxide in ischaemic tissue (McCord 1985). The production of ODFRs by xanthine oxidase and the neutrophil enzymes, NADPH oxidase and myeloperoxidase, has been discussed in section 1.1 part b.

The leakage of electrons from electron transport chains is the most important source of superoxide in aerobic cells (Halliwell and Gutteridge 1985). In the mitochondrial electron transport chain, more than 95% of oxygen is reduced to water by cytochrome oxidase. However, approximately 1-2% of the electron flow leaks into the oxygenase pathway to form superoxide radicals (Grisham and Granger 1989). The NADH-Coenzyme Q reductase complex and reduced forms of Coenzyme Q are the main sites of electron leakage (Halliwell and Gutteridge 1985). The microsomal mixed function oxidase system (MFO) is another electron transport chain, whose purpose is the inactivation or detoxification of reactive endogenous substances (Grisham and Granger 1989). Sometimes the MFO system converts relatively nontoxic compounds to toxic ones. For example, carbon tetrachloride is converted to the damaging trichloromethyl radical (Halliwell 1994).

Hydroxyl radical and hydrogen peroxide are formed as byproducts of the degradation of catecholamines by monoamine oxidase (Hearse *et al* 1986) and peroxyl and hydroxyl radicals are formed during the metabolism of arachidonic acid via the cyclo-oxygenase and lipoxygenase pathways (Flitter 1993). The most significant self generation of ODFRs is the formation of hydroxyl radicals from superoxide and hydrogen peroxide via the Fenton reaction (Hearse *et al* 1986) (see Figure 1.2).

(c) General Properties of Free Radicals

Radicals are unstable, highly reactive molecules due to the presence of the "extra" unpaired electron which is readily available to partake in chemical reactions (VanSteenhouse 1987).

The most potent ODFR is the hydroxyl radical (Parks and Granger 1983, Halliwell 1994). This radical is poorly diffusible and tends to cause damage at the closest target to its site of formation (Weiss 1986). It has an extremely short half life of 10^{-9} seconds (Pryor 1986). Hydroxyl radicals are capable of initiating lipid peroxidation (Kirschner and Fantini 1994).

Superoxide radical has good potential to react but rapid dismutation to hydrogen peroxide limits its reactivity (Weiss 1986). It can cross plasma membranes via anion channels, can directly permeate lipid bilayers and can gain access to intracellular sites as the protonated form of superoxide (perhydroxyl HO_2^*), which is uncharged (Weiss 1986).

Hydrogen peroxide is formed from the nonenzymatic or superoxide dismutase catalysed dismutation of superoxide (Halliwell 1994). It has good diffusion ability, and can freely cross cell membranes (Diplock 1994). It is a strong oxidant but its relatively slow reaction rate with organic substances limits its reactivity (Weiss 1986). The half lives of superoxide and hydrogen peroxide vary depending on the activity of the enzymatic scavengers, superoxide dismutase, catalase and glutathione peroxidase, and therefore are difficult to determine (Pryor 1986).

Lipid peroxyl and alkyl radicals are very reactive, with short half lives of 7 seconds and 10^{-6} seconds respectively (Pryor 1986).

(d) Radical Reactions And Their Effects

Oxygen derived free radicals have both beneficial and detrimental effects. The role of ODFRs, particularly superoxide radical, in bacterial killing and destruction of viral particles by phagocytes is perhaps the best known beneficial effect (Hitt 1990). Superoxide radical is also produced by lymphocytes and fibroblasts and is thought to play a role in intercellular signalling and growth regulation (Halliwell 1994). It can stimulate the growth of fibroblasts and epithelial cells *in vitro*, and may play a role in fibrosis and wound healing (Winrow *et al* 1993).

However, the destructive effects of ODFRs predominate, and all biomolecules appear to be susceptible to the deleterious effects of ODFRs (Cheeseman and Slater 1993).
The destructive effects of ODFRs include:

1. Peroxidation of lipid membranes (Halliwell 1994)
2. Oxidation of cell membrane proteins (receptors, enzymes, transduction systems) (Hitt 1990)
3. Fragmentation of hyaluronic acid, collagen and intercellular binding proteins (Kirschner and Fantini 1994)
4. Damage to deoxyribonucleic acid (DNA) (Cerutti 1985)
5. Chemoattraction (Zimmerman *et al* 1990).

When any radical reacts with a nonradical, another radical is formed, causing a chain reaction of radical formation (Hitt 1988). The chain reaction will continue until a radical reacts with another radical to form a non radical (Weiss 1986), or is scavenged by an antioxidant (see section 1.2 part e).

Lipids, particularly those containing polyunsaturated fatty acids (PUFAs), are highly susceptible to ODFR induced damage (Cheeseman and Slater 1993). Radicals with strong oxidising capabilities (eg hydroxyl radical), can peroxidise lipid membranes by extracting a hydrogen ion from a PUFA side chain in the cell membrane (Halliwell 1994) leaving an unpaired electron to form lipid alkyl radical (Weiss 1986). Rearrangement of the double bond in the lipid alkyl radical forms a conjugated diene, which rapidly reacts with oxygen to form a lipid peroxyradical (Kirschner and Fantini 1994) (see Figure 1.6). Peroxyradicals attack and remove a hydrogen ion

from adjacent fatty acid side chains to form lipid hydroperoxides and another alkyl radical, and this acts to propagate the reaction (Halliwell 1994) Lipid hydroperoxides are directly toxic and inactivate enzymes by oxidising susceptible amino acid residues (Weiss 1986). Lipid hydroperoxides can also break down to form aldehydes which are extremely destructive and can diffuse to other parts of the cell to cause further damage (Cheeseman and Slater 1993), or can react with iron or iron complexes to make peroxy- or alkoxyradicals which induce further lipid peroxidation (Kirschner and Fantini 1994).

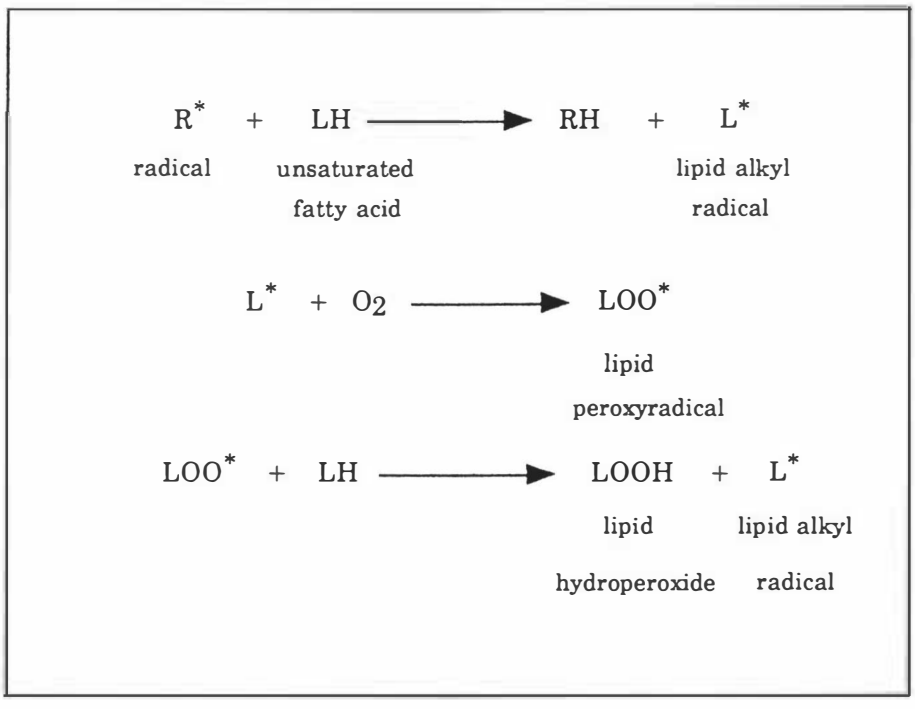


Figure 1.6 The Chain Reaction of Lipid Peroxidation by Oxygen Derived Free Radicals (from Rochat 1991)

Cell membranes are composed of a lipid bilayer with proteins attached, embedded or traversing the lipid (Halliwell and Gutteridge 1985). Controlled lipid peroxidation is a normal cellular process for prostaglandin and leukotriene synthesis, pinocytosis and controlled disassembly of the intracellular membrane (Rochat 1991) Uncontrolled, excessive lipid peroxidation by ODFRs leads to cell membrane and intercellular disruption, altered membrane permeability, release of toxic ions (Fe^{2+}, Ca^{2+}, Cu^{2+}), activation of phospholipase and kinase, disruption of lysosomes

and mitochondrial respiratory failure (Hitt 1990). Cellular destruction is the result (Cerutti 1985). All cell membranes are susceptible to ODFR damage, but those containing large quantities of PUFA, such as mitochondrial and microsomal membranes, are most susceptible (Weiss 1986).

Peroxyradicals also attack cell membrane proteins, such as receptors, enzymes and transduction systems, causing cellular dysfunction (Hitt 1990). Oxygen derived free radicals irreversibly inhibit the Na^+K^+ATPase pump, and alter its structure so it becomes more susceptible to degradation by proteolysis (Huang *et al* 1992). Generation of transmembrane peroxyclusters forms new membrane permeability channels which act as channels for calcium (Bindoli 1988). The rise in intracellular calcium disturbs cellular processes, as intracellular calcium concentrations must be finely controlled for normal cell function (Weiss 1986).

Hydroxyl radicals can damage hyaluronic acid, collagen and intercellular binding proteins like fibronectins, leading to basement membrane and cell junction separation (Hitt 1988).

Free radicals, particularly the hydroxyl radical, can react with the sulphydryl and deoxyribose groups on deoxyribonucleic acid (DNA), inducing breaks in single stranded DNA (Hitt 1988), and causing mutations (Cerutti 1985). Damage to DNA also results in altered protein structure and enzyme inactivation (Flaherty and Weisfeldt 1988).

Oxygen derived free radicals contribute to the inflammatory reaction produced following reperfusion of ischaemic tissues, by promoting the attraction and migration of inflammatory cells into the tissues (McCord 1983). It has been shown that neutrophil influx during reperfusion in *in vivo* intestinal ischaemia-reperfusion injury, can be attenuated by pretreatment with allopurinol and free radical scavengers (Grisham *et al* 1986, Zimmerman *et al* 1990, Kubes and Granger 1992). Hydrogen peroxide can react with iron or haemoglobin in the extracellular fluid to produce a chemotactant, although the precise substance is unknown (Zimmerman *et al* 1990). Hydrogen peroxide can also hydrolyse complement C5 to form a C5a-like chemotactic factor (Vogt *et al* 1989, Granger and Korthuis 1995). In the presence of human plasma, superoxide has

been shown to be chemotactic (Petrone *et al* 1980), and it is thought that superoxide reacts with lipoproteins or other substances in the extracellular fluid, to produce chemotactic substances (Kubes and Granger 1992). Oxidant induced damage to tissue may result in the release of proteases, that also activate the complement system and contribute to chemotaxis (Grisham *et al* 1986).

Infiltration of neutrophils into the tissue requires interactions between membrane-associated adhesive glycoproteins on neutrophils and endothelial cells, which promote neutrophil rolling and adhesion (Moore *et al* 1994, Menger 1995). These adhesive glycoproteins include L-selectin and CD11/CD18 on neutrophils, and P-selectin and intercellular adhesion molecule-1 (ICAM-1) on endothelial cells (Granger and Korthuis 1995). Hydrogen peroxide increases the expression of these glycoproteins, possibly via the enhanced production of platelet activating factor (Suzuki *et al* 1991, Patel *et al* 1991, Granger and Korthuis 1995). Expression of ICAM-1 and P-selectin is also enhanced by cytokines such as tumour necrosis factor and interleukin-1, and by histamine and thrombin (Toledo-Pereyra and Suzuki 1994, Granger and Korthuis 1995). In addition, superoxide may inactivate nitric oxide, an important endogenous inhibitor of leukocyte adherence in post capillary venules (Kubes and Granger 1992).

Oxygen derived free radicals also have effects on the cardiovascular and haematological systems. They have been shown to decrease the contractility of the left ventricle in rabbits, and reduce heart rate and cardiac output in dogs (Prasad *et al* 1993). Hydrogen peroxide causes vasoconstriction of cerebral and pulmonary arteries by depressing nitric oxide-dependent smooth muscle relaxation within the blood vessels, and via the stimulation of arachidonic acid metabolism, leading to the production of vasoconstricting metabolites (Whorton *et al* 1985, Katusic *et al* 1993). Oxygen derived free radicals also induce platelet aggregation and activation (Yoshikawa 1990a, Ikeda *et al* 1994).

It can be seen that oxidant attack on lipoprotein membranes initiates a complex cascade of events, leading to the formation of more radicals, long lived toxic byproducts and biologically active inflammatory mediators that propagate damage beyond the confines of the original focus. The end result is widespread cellular injury and inflammation. The estimated rate of ODFR

production at physiological temperature and pH during reperfusion of ischaemic tissue is sufficient to cause *in vitro* cytotoxicity and increased vascular permeability (Parks *et al* 1988). Oxygen derived free radicals have been shown to cause *in vivo* mucosal damage (Granger *et al* 1981), and increase the permeability of the intestinal capillaries (Parks and Granger 1983, Granger *et al* 1981) in experimental intestinal ischaemia, probably secondary to lipid peroxidation or basement membrane destruction.

Oxygen derived free radicals have been implicated in the pathogenesis of autoimmune disorders (Hitt 1988), pancreatitis (Sarr *et al* 1987), acute respiratory distress syndrome (Nielsen *et al* 1996), irreversible shock (Yoshikawa 1990b), myocardial infarction and secondary arrhythmias (Chamber *et al* 1985, Svendsen and Bjerrum 1992), and of course, ischaemia-reperfusion injury (Hitt 1990).

(e) Defences Against Oxygen Derived Free Radicals

Given the damaging effects of ODFRs, it is not surprising that the body is equipped with a defence system to inactivate the relatively small quantities of radicals generated on a day to day basis.

An antioxidant is a substance which can delay or prevent the oxidation of a substrate (Diplock 1994). An antioxidant may utilise one or more of the following mechanisms of action:

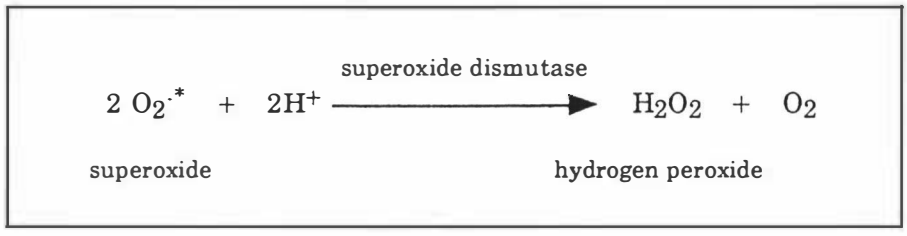
1. Elimination of superoxide (superoxide dismutase)
2. Decomposition of hydrogen peroxide and lipid peroxides
3. Scavenging of free radicals, thereby preventing initiation of chain reactions
4. Sequestration of metal catalysts to prevent radical formation
5. Repair of damage (Forsyth and Guilford 1995).

Prevention of radical formation is an additional important defence mechanism. The use of metabolic pathways such as mitochondrial cytochrome oxidase, that make relatively little ODFRs compared with the alternative metabolic pathways, limits ODFR production (Fridovich

and Freeman 1986). The presence of cholesterol within the lipid membrane structure interrupts the chain reaction of radical induced lipid peroxidation (Halliwell and Gutteridge 1985).

The major enzymes involved in radical inactivation are superoxide dismutase, catalase and glutathione peroxidase (Diplock 1994).

Superoxide dismutase (SOD) is an intracellular enzyme (Rochat 1991), found in both the mitochondria and cytosol (Halliwell 1994). It is a metalloprotein which catalyses the chemical reaction to destroy superoxide (Hitt 1990):

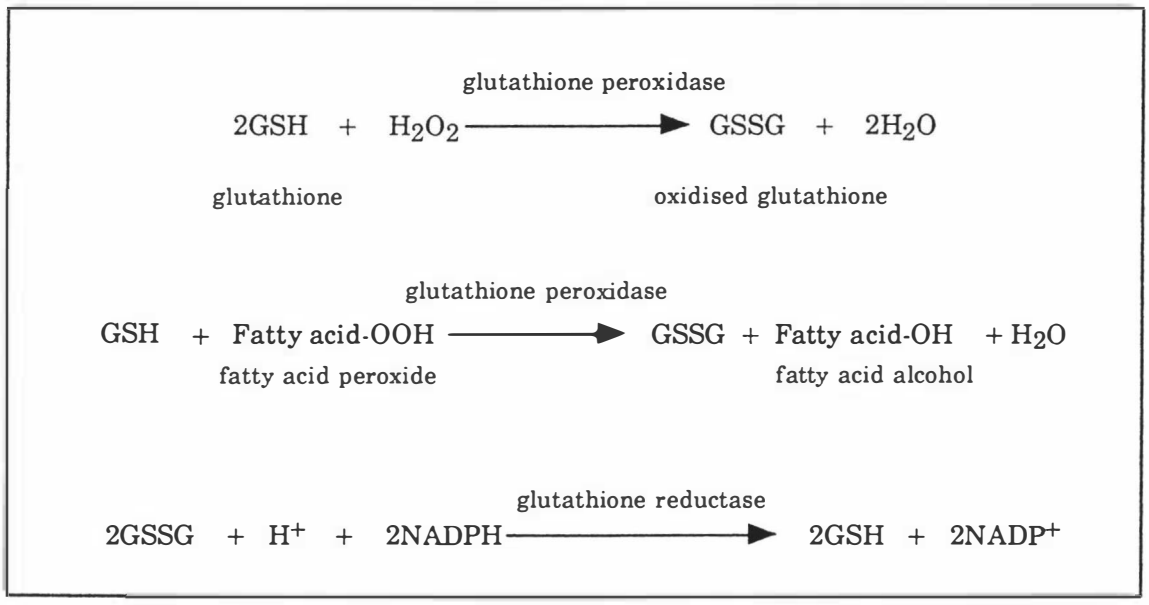


Superoxide dismutase is found in many tissues of the body, with large amounts in the pancreas (Parks et al 1983), liver, kidney and adrenal gland (Diplock 1994). SOD cannot penetrate cells easily (Guilford 1990) and has a short half life of just 6 minutes after intravenous injection (McCord 1986).

Catalase is an intracellular haemoprotein enzyme found in subcellular organelles called peroxisomes (Halliwell and Gutteridge 1985). It catalyses the conversion of hydrogen peroxide to water and oxygen (Fridovich and Freeman 1986).

High concentrations of catalase are found in the liver and red blood cells, with less in the brain, heart and skeletal muscle (Diplock 1994). Catalase also has a short half life of 4 minutes following intravenous injection (McCord 1986).

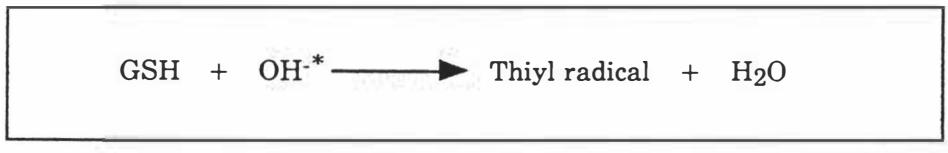
Glutathione peroxidase is a selenium containing intracellular enzyme (Meister 1982) found within the cytosol and mitochondria (Diplock 1994). It catalyses the degradation of hydrogen peroxide and fatty acid peroxides, using glutathione (GSH) as a substrate. Oxidised glutathione (GSSG) is reconverted to GSH by the enzyme glutathione reductase. The reactions are summarised below (Halliwell 1994):



Glutathione peroxidase is probably more important than catalase in the removal of hydrogen peroxide (Halliwell 1994).

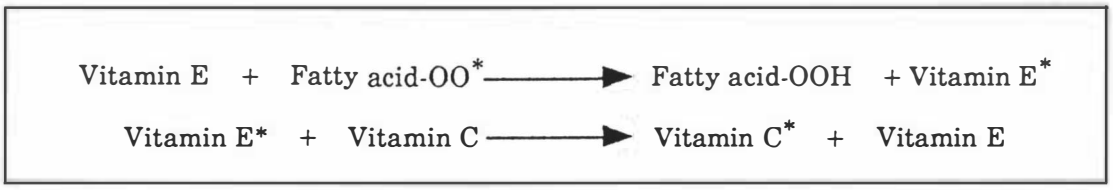
Other antioxidants include:

• *glutathione (GSH)* - is an amino acid that is a direct free radical scavenger, in addition to its participation in the reactions catalysed by glutathione peroxidase shown above (Guilford 1990). The reaction of GSH with hydroxyl radical produces a thiyl radical, which is less reactive than hydroxyl, but still able to initiate reactions and must also be scavenged (Diplock 1994).



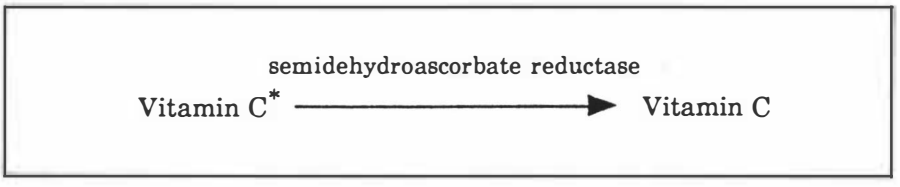
• *alpha-tocopherol (Vitamin E)* - is an important membrane bound antioxidant (Diplock 1994). Vitamin E consists of eight tocopherols, of which alpha-tocopherol is the most important

antioxidant. It is incorporated into the membrane lipid bilayer to inhibit peroxidation and the extracellular release of hydrogen peroxide (VanSteenhouse 1987). Alpha-tocopherol reacts with lipid peroxyl radicals, becoming oxidised to alpha-tocopheroxy radical, which is relatively stable and cannot peroxidise lipid (Cheeseman and Slater 1993). Alpha-tocopherol is efficiently regenerated in a reaction involving Vitamin C (Diplock 1994).



Alpha tocopherol can scavenge singlet oxygen (Weiss 1986), and may also specifically inhibit the phagocytic cell NADPH-oxidase enzyme system (Butterick *et al* 1983).

• *ascorbic acid (Vitamin C)* - scavenges superoxide, hydroxyl, hypochlorous acid, nitroxide, thiyl and sulphonyl radicals (Halliwell 1994) and singlet oxygen (Diplock 1994). It is synergistic with alpha-tocopherol due to its ability to regenerate oxidised alpha-tocopherol by donating a hydrogen ion (Niki 1987). It is regenerated via the enzyme semidehydroascorbate reductase (Weiss 1986).



• *β-carotene (Pro-Vitamin A)* - is synergistic with alpha-tocopherol, but the mechanism is unknown. It scavenges radicals, especially singlet oxygen (Diplock 1994).

• *Coenzyme Q10 (Ubiquinone)*- may protect cell membranes from radical damage (Diplock 1994).

• *Uric acid* - is a powerful scavenger of peroxyl and hydroxyl radicals, lipid hydroperoxides, hypochlorous acid and singlet oxygen (Wagner *et al* 1986, Ames *et al* 1981)

• *Bilirubin* - scavenges superoxide and hydroxyl radical (Rangan and Bulkley 1993).

• *Albumin* - scavenges fatty acid peroxides and hypochlorous acid (Rangan and Bulkley 1993).

•*Cysteine* (Anderson *et al* 1987) *Hypotaurine* and *Taurine* (Arouma *et al* 1988) are also antioxidants.

Superoxide dismutase, catalase and glutathione peroxidase are somewhat limited by their intracellular location. If ODFRs manage to diffuse out of the cell, great tissue damage can result (Salin 1975). In this situation, extracellular defence mechanisms including the scavengers, alpha-tocopherol, Vitamin C, cysteine and albumin, and the sequestration of metal catalysts, particularly iron, are extremely important. Transferrin and lactoferrin bind circulating iron (Halliwell and Gutteridge 1985), haptoglobin binds haemoglobin and ceruloplasmin maintains iron in the ferric form (Forsyth and Guilford 1995). Ferritin binds tissue iron (Ranglan and Bulkley 1993). Oleic acid is a monounsaturated fatty acid that inhibits lipid peroxidation, probably by complexing with transition metals (Diplock 1994).

The final line of defence is the repair process (Fridovich and Freeman 1986). Normal membrane turnover releases damaged lipids, oxidised proteins are destroyed by proteases, and oxidised methionine is repaired by the enzyme methionine sulphoxide (Halliwell 1994).

Oxygen derived free radicals can overwhelm the body's defence mechanisms by several means. Depletion of scavengers and saturation of antioxidant enzymes will occur if rates of lipid peroxidation or ODFR production are extremely high (Kirschner and Fantini 1994). Some ODFRs inactivate antioxidant enzymes, for example superoxide can inactivate catalase, and hydrogen peroxide can inactivate superoxide dismutase. Finally, compartmentalisation of the defences relative to the site of attack may limit their efficacy to prevent oxidative damage (Weiss 1986).

Antioxidants used therapeutically include ODFR scavengers, such as mannitol and DMSO (Badylak 1986) and iron chelators, such as desferrioxamine (Lantz and Badylak 1989). Nonglucocorticoid aminosteroids (lazaroids), currently only experimental drugs, inhibit lipid

peroxidation and may scavenge free radicals (Hall 1988). Beef liver superoxide dismutase is available for parenteral use (Orgotein (Palosein™)), but its efficacy is limited by rapid removal from the body by renal excretion, and its inability to enter cells (Huber *et al* 1980). Polyethylene glycol-enzyme conjugates of superoxide dismutase and catalase have been developed to reduce the rate of elimination from the body (Viau *et al* 1986), and the enzyme has been incorporated into liposomes which enables it to enter cells (Jamieson *et al* 1986). Allopurinol, a xanthine oxidase inhibitor, acts as a free radical scavenger at extremely high concentrations in the extracellular fluid (>1000 μmol/l) (Klein *et al* 1996). However this is unlikely to be a clinically applicable therapeutic strategy as concentrations of only 10-20 μmol/l are reached following administration at recommended dosages, and at this concentration no scavenging occurs (Zimmerman *et al* 1988).

1.3 Xanthine oxidase

(a) Structure and Function

Xanthine oxidase (xanthine:oxygen oxidoreductase E.C.1.2.3.2) is a metallo-flavoprotein containing flavin adenine dinucleotide (FAD), molybdenum and iron, in a ratio of 2:2:8 (Huber *et al* 1996, Fried and Fried 1974). Under the International Union of Biochemistry Nomenclature, xanthine oxidase is classified into the oxidoreductase group of enzymes, which catalyse redox (reduction-oxidation) reactions (Mayes 1988). Xanthine oxidase can be subclassified into the group of aerobic dehydrogenases, which can use oxygen or artificial substrates as electron acceptors (Mayes 1988).

Four forms of xanthine oxidase are found in mammals (Della Corte and Stirpe 1972), and their structures are similar among mammals (Sarnesto *et al* 1996):
(i) Xanthine dehydrogenase is a large protein consisting of two 150,000 Dalton subunits (Saksela and Raivio 1996). It can only use NAD^+ as an electron acceptor (Parks and Granger 1986a).
(ii) Intermediate Dehydrogenase/Oxidase is an intermediate between xanthine dehydrogenase and reversible xanthine oxidase, and is capable of using NAD^+ or oxygen as an electron acceptor (Parks and Granger 1986a).
(iii) Irreversible and (iv) reversible xanthine oxidase can only use oxygen as an electron acceptor (Kirschner and Fantini 1994). Irreversible xanthine oxidase differs from reversible xanthine oxidase, in that it lacks a 20,000 Dalton polypeptide fragment at each subunit (Parks and Granger 1986a).

The structural difference between the dehydrogenase, the intermediate dehydrogenase/oxidase, and the oxidase forms, is an alteration in the protein conformation around the FAD site, which alters the redox potential of flavin, and the reactivity of FAD with electron acceptors (Nishino 1994), however the precise nature of the structural difference is currently unknown (Bray *et al* 1996).

In nonischaemic tissues, the predominant form of the enzyme is the dehydrogenase form (Stirpe and Della Corte 1969), with xanthine oxidase comprising only 10-20% of the total xanthine oxidase plus dehydrogenase activity (Engerson *et al* 1987, McKelvey *et al* 1988, Parks *et al* 1988). The majority of the xanthine oxidase in nonischaemic tissues (approximately 85%), is in the irreversible form (McKelvey *et al* 1988).

The four forms of the xanthine oxidase system can be interconverted by several mechanisms. Conversion of xanthine dehydrogenase to reversible xanthine oxidase occurs through thiol group oxidation (Saksela and Raivio 1996), and this may occur directly, or indirectly via the intermediate dehydrogenase/oxidase form (Stirpe and Della Corte 1969). Reversible xanthine oxidase can be converted back to the dehydrogenase form by thiol reducing agents, such as dithiothreitol and dithioerythritol (Della Corte and Stirpe 1972). Conversion of the reversible form of xanthine oxidase, to the irreversible form, occurs by the cleavage of the 20,000 Dalton fragment from each subunit, presumably catalysed by a calcium-dependent proteases (Saksela and Raivio 1996, Nielsen *et al* 1996). Conditions favouring the conversion of xanthine dehydrogenase, to xanthine oxidase, prevail in ischaemic tissues (Sarnesto *et al* 1996).

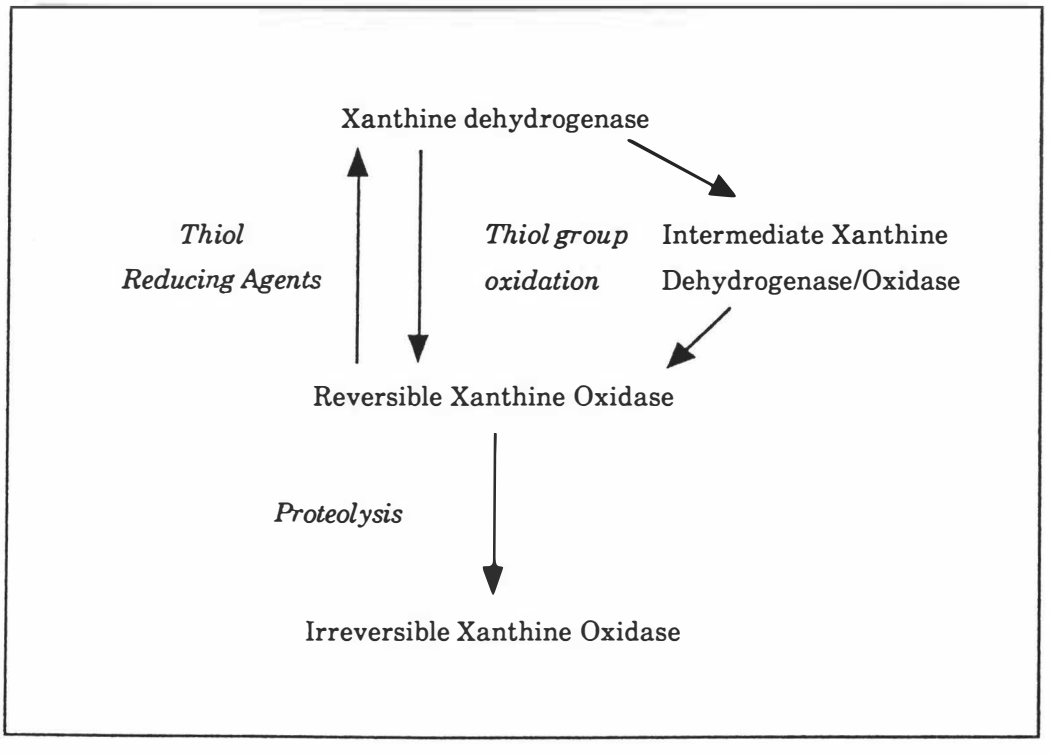


Figure 1.7 The Relationship Between and Interconversion of Xanthine Dehydrogenase, Intermediate Xanthine Dehydrogenase/Oxidase, and Xanthine Oxidase.

Xanthine dehydrogenase and oxidase have four redox active sites (Fried and Fried 1974). Purines or other substrates interact at the molybdenum site (Bray *et al* 1996), and the electron acceptors (oxygen or nicotinamide adenine dinucleotide (NAD^+)) interact at the FAD site (Hille and Massey 1981, Bray *et al* 1996). The enzyme can oxidise a range of substrates including purines, pyrimidines, pteridines, azopurines, cytochrome C, heterocyclic compounds, oxygen, NAD^+ and ferricyanide (Parks and Granger 1986a). The primary role of the xanthine oxidase system is in the metabolism of purines (Fried and Fried 1974, Sarnesto *et al* 1996).

Adenine and guanine are the the two major purines in the body. Hypoxanthine and xanthine are also purines, being intermediaries in the metabolism of adenine and guanine. Other minor purines are 5 methylcytosine, 5 hydroxy-methylcytosine, N^6-methyladenine, N^7-methylguanine and N^6,N^6-dimethyladenine (Rodwell 1988). Purines are biologically important as monomeric precursors of DNA and RNA, and are constituents of adenosine triphosphate (ATP), cyclic adenosine monophosphate (cAMP) and the co-enzymes flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) (Rodwell 1988). Nucleosides (purine or pyrimidine bonded to a sugar eg adenosine) nucleotides (nucleosides in which the purine or pyrimidine is phosphorylated to a sugar eg adenosine monophosphate (AMP)), nucleoproteins and ingested purines are degraded to uric acid by a series of reactions catalysed by enzymes (Rodwell 1988). Xanthine dehydrogenase and xanthine oxidase catalyse the final reactions in this pathway, which are considered to be the rate limiting step (Fried and Fried 1974). These reactions lead to the conversion of hypoxanthine to xanthine (Roussos 1987), followed by the conversion of xanthine to uric acid (McCord 1985), yielding hydrogen peroxide and superoxide radicals as byproducts (Kirschner and Fantini 1994) (see Figure 1.8). Under aerobic conditions, these reactions generate substantial quantities of superoxide radical and hydrogen peroxide (Fridovich 1985).

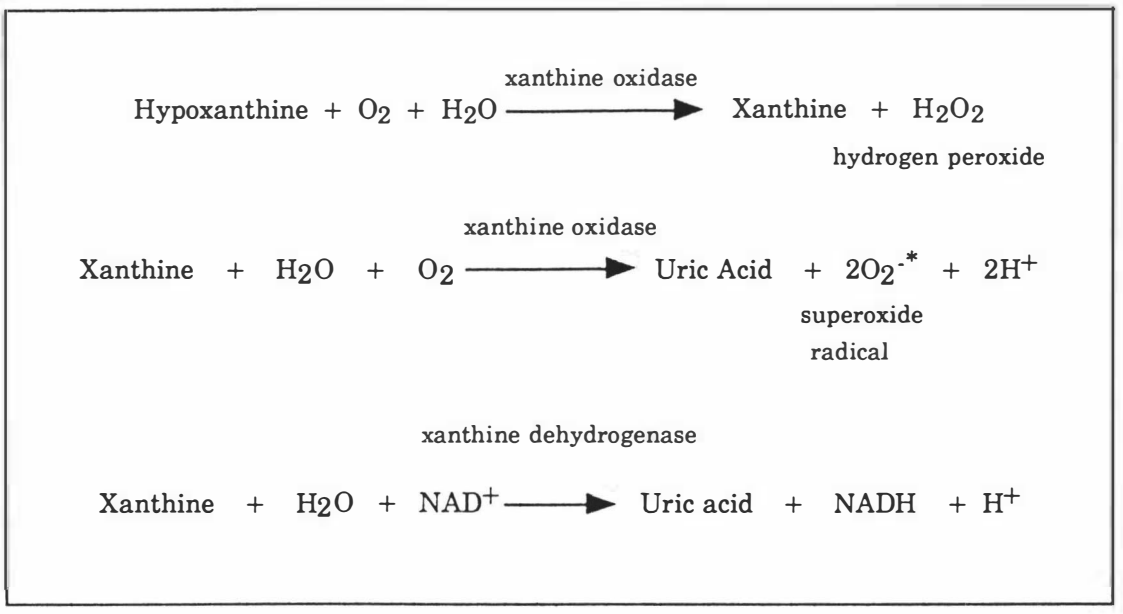


Figure 1.8 Formation of Uric Acid from Hypoxanthine, Catalysed by the Xanthine Oxidase System

Xanthine oxidase has several other physiological functions. It plays a role in iron absorption in the small intestine, by oxidising dietary iron from the ferrous state (Fe^{2+}) to the ferric state (Fe^{3+}) within the intestinal mucosa, facilitating the absorption of iron (Topham *et al* 1982a). Xanthine oxidase also mobilises iron from the liver by promoting the release of iron ferritin (Topham *et al* 1982b). It oxidises pteridines, heterocyclic bases and participates in the oxidation of sulphydryl (thiol) groups in glutathione, fatty acids, phospholipids, amino acids and epinephrine (Parks and Granger 1986a), catalyses the oxidation of aldehydes to carboxylic acids (Fridovich 1985) and plays a role in alcohol metabolism (Fried and Fried 1974).

In addition, a role for xanthine oxidase in antimicrobial defence, similar to NADPH oxidase in phagocytes is possible. It has been proposed that xanthine dehydrogenase in the endothelial cell cytoplasm is released following endothelial injury by microbes, and is converted to the oxidase form in the oxygen rich environment of blood. Xanthine oxidase can oxidise substrates in the blood, leading to the formation of oxygen derived free radicals which have been suggested to provide oxidative defence (Jarasch *et al* 1986).

(b) Distribution of xanthine oxidase

Xanthine oxidase is found in many species ranging from bacteria and molluscs, to birds and mammals (Al-Khalidi and Chaglassian 1965). The amount of xanthine oxidase activity varies between species and between different organs within a species (Al Khalidi and Chaglassian 1965, Krenitsky *et al* 1974).

Al-Khalidi and Chaglassian (1965) determined the xanthine oxidase activity in the blood and serum of twenty two species (16 mammals and 6 non-mammals). The dog, cow, guinea pig, rat, mouse and badger showed the highest serum xanthine oxidase activities (see Table 1.1). Among the mammalian species, humans, cats, goats, camels, sheep and pigs had the lowest serum xanthine oxidase activities. There was no detectable serum xanthine oxidase activity in humans, sheep and pigs. In this study, only two or three individuals from each of the species were examined. The average concentration of xanthine oxidase in the serum from three healthy dogs was 44 mU/litre.

The serum xanthine oxidase activity in four avian species tested was minute, but xanthine oxidase was present within the erythrocytes. Mammalian erythrocytes did not contain any measurable xanthine oxidase activity (Al-Khalidi and Chaglassian 1965). The observation that the blood xanthine oxidase activity of mammals is approximately one half of that in the serum probably reflects the volume of blood occupied by the erythrocytes, which lack xanthine oxidase.

Animals with high serum xanthine oxidase tend to have high pulmonary xanthine oxidase. Comparison of the xanthine oxidase activity in blood from the left and right ventricles showed a higher activity in the left ventricular blood, suggesting that xanthine oxidase may leak from the pulmonary tissue into the bloodstream (Al-Khalidi and Chaglassian 1965) (see Table 1.2).

Species	Xanthine oxidase activity in mU/l blood/serum or mU/gram tissue		
	Blood	Serum	Lung
Mouse	-	423.0	-
Badger	-	207.0	-
Cow	62.0	142.0	48.0
Guinea pig	63.0	125.0	-
Rat	52.0	101.0	53.0-180.0
Dog	23.0	44.0	21.0-24.0
Horse	-	40.0	-
Donkey	-	29.0	-
Chicken	16.0	1.1	-
Tortoise	15.0	0.3	-
Rabbit	14.0	24.0	-
Fox	-	12.0	-
Goat	0.0	1.0	-
Duck, Goose,Pigeon	0.0	0.9	-
Camel	-	0.7	-
Cat	0.3	0.6	6.0
Human	0.0-34.6	0.0	0.0-110.0
Pig	0.0	0.0	-
Sheep	0.0	0.0	0.0

Table 1.1 Blood, Serum and Pulmonary Xanthine Oxidase Activity in Various Species
Data derived from Al-Khalidi and Chaglassian 1965, Krenitsky *et al* 1974 and Ramboer *et al* 1969

	Serum Xanthine Oxidase mU/l	
	Right Ventricle	Left Ventricle
Dog 1	26.4 ± 3.6	32.8 ± 5.1
Dog 2	8.4 ± 2.1	11.2 ± 1.9

Table 1.2 Serum Xanthine Oxidase Activity (mU/l) in the Blood from the Ventricles of 2 Dogs
Derived from Al-Khalidi and Chaglassian 1965

Tissue xanthine oxidase activities in the dog, cat, rat, mouse, guinea pig, rabbit, sheep, cow and humans have also been measured by a variety of techniques. Al-Khalidi and Chaglassian (1965) used a radioisotopic assay, and Ramboer (1969) Krenitsky *et al* (1974) used a spectrophotometric method (see Tables 1.3 and 1.4) Their findings showed that the highest

quantity of xanthine oxidase activity was consistently found in the intestine, liver and lung. In most species, including the dog, the intestine contains the most xanthine oxidase activity. A gradient of xanthine oxidase activity has been recognised within the small intestine of the rat and cat, with greater xanthine oxidase activity present in the proximal ileum compared with the distal ileum, and the duodenum containing more xanthine oxidase activity than the proximal ileum (Parks *et al* 1988). Humans have higher xanthine oxidase activity in the liver than in other organs (Krenitsky *et al* 1974) and generally have relatively low activities compared with other mammals (Parks and Granger 1986a, Sarnesto *et al* 1996).

TISSUE	XANTHINE OXIDASE ACTIVITY mU/gram		
Author	**Al-Khalidi***	**Krenitsky***	**Ramboer***
Intestinal mucosa	24	-	-
Intestine (whole)	19	140-410	187
Liver	19	56-240	96
Lung	24	-	21
Kidney	-	<10-36	-
Thyroid	2.5	-	-
Colon	2.1	-	-
Spleen	2.1	-	84
Skin	1.5	-	-
Mesentery	1.4	-	-
Adipose	1.2	-	-
Uterus	0.8	-	-
Bone marrow	0.75	-	-
Adrenal	0.71	-	-
Ovary	0.6	-	-
Heart	0.3	-	0
Pancreas	0.3	-	-
Muscle	0.2	-	-
Brain	0.07	-	-

Table 1.3 Xanthine Oxidase Activity of Various Tissues In Dogs

*Data derived from Al-Khalidi and Chaglassian 1965, Krenitsky *et al* 1974 and Ramboer *et al* 1969

TISSUE	XANTHINE OXIDASE ACTIVITY mU/GRAM					
	DOG	CAT	SHEEP	COW	MAN	RAT
Intestinal mucosa	24.0	19.0	-	-	-	-
Intestine (whole)	19.0	30.0-200.0	0.1	12.0	10.0-190.0	530.0-950.0
	140.0 -410.0				56.0	405.0
	187.0					
Liver	19.0	17.0	12.0	29.0	31.0-220.0	330.0-720.0
	56.0-240.0	190.0-430.0		58.0	10.0-150.0	120.0-3000.0
	96.0					
Lung	24.0	6.0	0.0	48.0	10.0-110.0	53.0-180.0
	21.0			51.0	0.0	30.0
Spleen	2.1	0.0	0.2	20.0	44.0	49.0-160.0
	84.0			270.0	2.0	140.0
Kidney	10.0-36.0	42.0-96.0	-	15.0	13.0-130.0	130.0-220.0
Colon	2.1	1.0	0.0	0.1	76.0	70.0
				35.0		
Pancreas	0.3	1.5	1.0	5.7	-	38.0
Heart	0.3	0.0	0.0	0.2	74.0	12.0
	0.0			6.0	0.0	
Brain	0.2	0.0	0.0	-	<20.0	4.0

Table 1.4 Xanthine Oxidase Activity in the Tissues of Various Species

Data Derived from Al-Khalidi and Chaglassian 1965, Krenitsky *et al* 1974 and Ramboer *et al* 1969

The activity of xanthine oxidase varies between the different cells types within the tissues. Most of the intestinal xanthine oxidase is found within the mucosa (Granger *et al* 1986), predominantly in the enterocytes (Sackler 1966, Granger *et al* 1986) and endothelial cells (Jarasch *et al* 1986). The enterocytes at the apical portion of the villi contain the most xanthine oxidase activity, with little or no activity present in the lamina propria, muscularis layer, brush border and goblet cells (Pickett *et al* 1970).

In other tissues, the liver, lung, and muscle (skeletal and cardiac), xanthine oxidase is predominantly found within the endothelial cells (Jarasch *et al* 1986). Hepatocytes (Ichikawa *et al* 1992), pancreatic acinar cells (McIndoe *et al* 1974), renal collecting duct epithelial cells (Parks and Granger 1986a, Sackler 1966), gastric, oesophageal and bronchial epithelial cells (Moriwaki *et al* 1996), the sarcolemma of skeletal muscle (Apple *et al* 1991), and the alveolar epithelial cells of the mammary gland (Jarasch *et al* 1988) also contain xanthine oxidase.

Ultrastructurally, xanthine oxidase is located within the cytosol, either freely, or within peroxisomes (Ichikawa *et al* 1992, Angermuller *et al* 1987).

Xanthine oxidase is also found in large quantities in the milk, but its function here is unknown (Fried and Fried 1974).

Antibodies to xanthine oxidase have been detected in sera from humans (Bruder *et al* 1984, Jarasch *et al* 1986, Harris *et al* 1993), cows, goats, guinea pigs, mice and rabbits (Bruder *et al* 1984). The concentration of the xanthine oxidase specific antibody was between 0.2 and 8% of the total immunoglobulin G, thus forming a significant proportion of the body's immunoglobulin. It is thought that the antibody is present due to self immunisation against xanthine oxidase which may leak from small endothelial lesions throughout life, rather than secondary to the consumption of cows' milk, as the immunoglobulin binds more strongly to endogenous xanthine oxidase than the bovine milk enzyme (Jarasch *et al* 1986).

In nonischaemic tissues, xanthine dehydrogenase is the predominant form of the enzyme (Stirpe and Della Corte 1969). Under hypoxic conditions, xanthine dehydrogenase can be converted to the oxidase form by sulphydryl oxidation and proteolysis, as described in part 1.3a. Conversion to both the reversible and irreversible oxidase forms occurs in all tissues except the small intestine, where all xanthine oxidase is irreversible (Waud and Rajagopalan 1976, Della Corte and Stirpe 1972). In the liver and kidney of the rat it has been shown that initial conversion is to the reversible oxidase form, with rises in irreversible xanthine oxidase activity lagging behind by approximately 2 hours (Engerson *et al* 1987).

The rate of conversion of xanthine dehydrogenase to oxidase in ischaemia-reperfusion injury is generally rapid. Conversion rates have varied between authors, the organs studied, and the different experimental conditions used. Two hours of normothermic ischaemia of the small intestine of rats and dogs results in an increase in the xanthine oxidase activity to approximately 50% and 85% of the total dehydrogenase plus oxidase activity, respectively (Parks *et al* 1988, Hossain *et al* 1995). In contrast, Roy and McCord (1982) reported complete conversion of xanthine dehydrogenase to oxidase in less than 1 minute of ischaemia of the rat

intestine, but it is thought that this experiment was flawed by artifactual conversion during processing.

McKelvey *et al* (1988) and Engerson *et al* (1987) found similar rates of conversion in the rat liver, with 50% conversion to xanthine oxidase occurring in 3.8 and 3.6 hours respectively. Yokoyama *et al* (1990) reported a much slower rate of conversion, in that 64% conversion to xanthine oxidase took 8 hours, but faster rates of conversion in the liver have also been reported. For example, Roy and McCord (1983) found that 50% conversion to the oxidase form occured after 1 hour of ischaemia at 25°C, and Stirpe and Della Corte (1969) found that conversion was virtually complete after 10 minutes of ischaemia at 37°C. Half times of conversion to xanthine oxidase at 37°C in rat kidney, lung and heart have been reported to be 6 hours, 14 hours and 7 hours respectively (Engerson *et al* 1987). Parks *et al* (1988) found more rapid rats of conversion, with the xanthine oxidase activity doubling in 30 minutes in the lung and kidney. Xanthine oxidase activity has been reported to double after only 8-10 minutes of ischaemia in the heart (Granger 1988). The conversion of xanthine dehydrogenase to reversible xanthine oxidase has been reported to occur within seconds upon the release of xanthine dehydrogenase into plasma (Yokoyama *et al* 1990, Arnold *et al* 1994).

c) Enzyme kinetics of xanthine oxidase

The collision theory of reaction states that:

1. In order to react, molecules must collide ie become within bond forming reach of one another.
2. For a collision to be productive, reacting molecules must have enough energy to overcome the energy barrier for reaction.

Factors which increase the frequency of the collision or kinetic energy increase the rate of reaction. Such factors are:

(*i*) *Temperature* - over a limited range of temperature, enzyme activity will increase as temperature increases to an optimum temperature, usually near body temperature. After this time, rate will decrease primarily due to enzyme denaturation (Rodwell 1988).

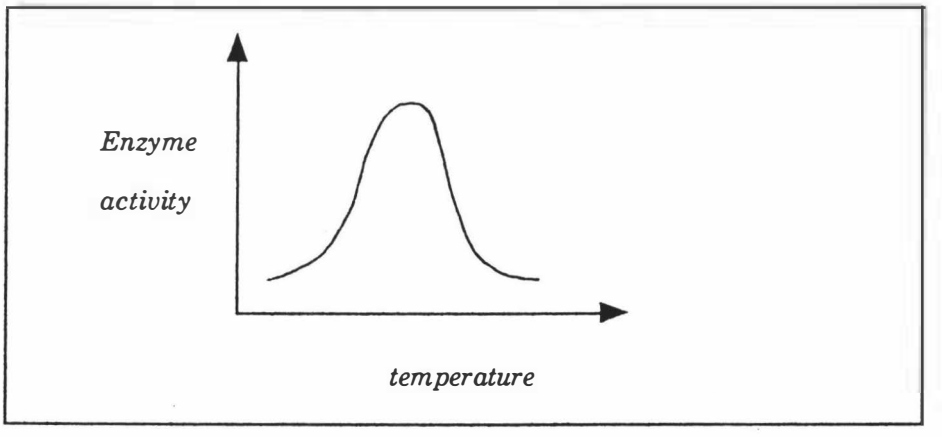


Figure 1.9 The Effect of the Temperature of Reaction on Enzyme Activity

The optimum temperature for xanthine oxidase in bovine milk, bovine intestine and equine serum is 37°C, 47-50°C and 28°C, respectively (Fried 1966, Roussos 1987, Lochner *et al* 1990) Equine serum xanthine oxidase activity was determined at temperatures between 20 °C and 40°C by Lochner *et al* (1990) and at room temperature (approximately 20°C) the activity was approximately 60% of the maximum activity. There have been no similar studies using canine serum.

(ii) pH - the pH affects the ionic state of the enzyme and substrate. Extremes of pH will also cause enzyme denaturation (Rodwell 1988). The optimum pH for xanthine oxidase activity varies between tissue types. The maximum activity of rat liver xanthine oxidase occured at a pH of approximately 8.35, and in bovine milk maximum activity occured at an approximate pH of 8.6 (Ramboer 1969). Equine serum xanthine oxidase activity is maximal at a pH of 7.8 (Lochner *et al* 1990). The activity of bovine intestinal xanthine oxidase depends upon the type of buffer used in the assay. For example, with 0.1M phosphate buffer the optimum pH is 7.5-7.7, but with 0.1M Tris buffer, it is 8.3-8.7 (Roussos 1987). Similar studies in dogs are lacking.

(iii) Substrate concentration - in general, as the substrate concentration increases, the frequency of collision increases, and hence the reaction rate increases. This occurs up to a point of maximum velocity (Vmax) and at this point, enzyme saturation occurs (see Figure 1.10) (Rodwell 1988). Substrate inhibition of xanthine oxidase occurs at high concentrations of xanthine and hypoxanthine (Fridovich 1985).

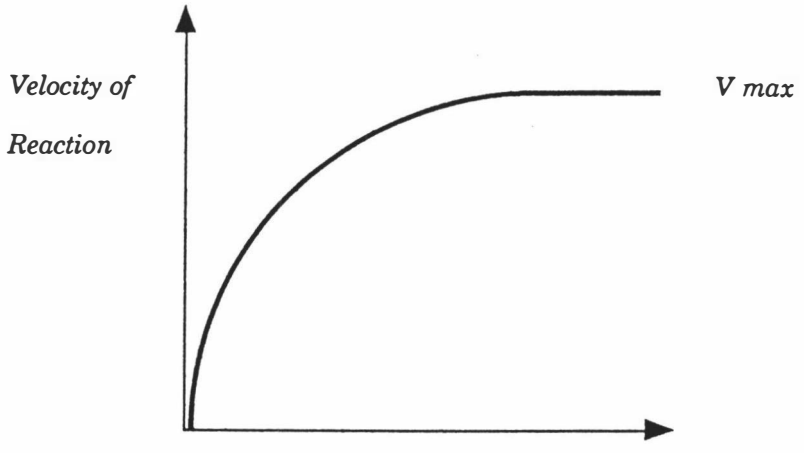


Figure 1.10 The Effect of Substrate Concentration on Reaction Velocity

(iv) Enzyme concentration - in appropriate conditions, reaction rate is proportional to enzyme activity (Rodwell 1988). The reaction rate was directly proportional to the concentration of milk xanthine oxidase at a concentration up to 2000mU per litre (Ramboer 1969). When enzymes catalyse a chemical reaction, they undergo physical changes during the reaction but revert to their original state when the reaction is complete. Two phases of product formation are seen in reactions requiring an enzyme catalyst. The burst phase corresponds to the conversion of all of the active enzyme molecules to the inactive form. The steady state phase that follows occurs as active enzymes are regenerated (Rodwell 1988) (see Figure 1.11)

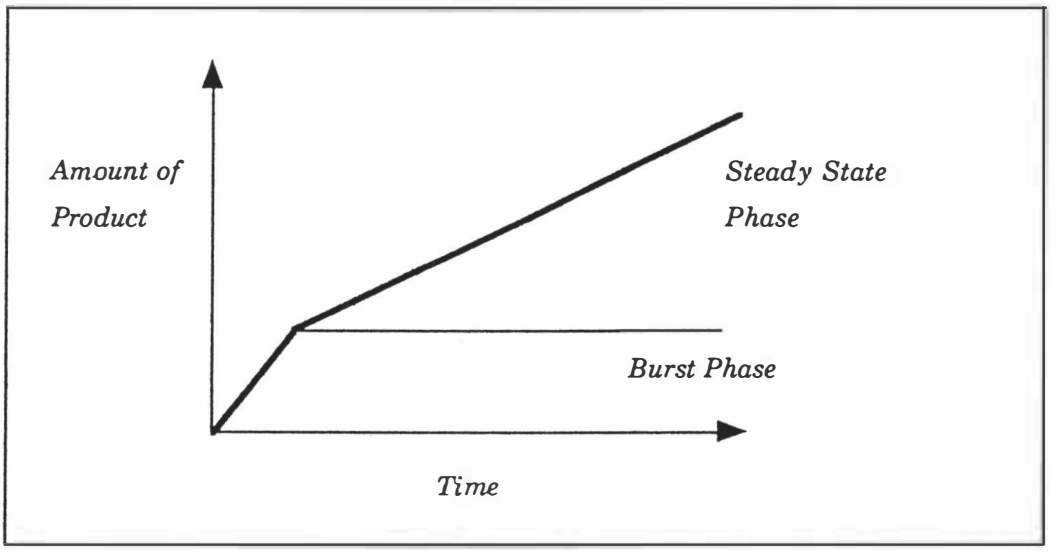


Figure 1.11 Kinetics of Reactions Catalysed by Enzymes

The production of uric acid by reactions catalysed by xanthine oxidase follows zero order kinetics after the burst phase - ie they are linear. Ramboer (1969) demonstrated this linear reaction when rat liver homogenate xanthine oxidase activity was measured over 7 hours at 25°C.

(v) Enzyme inhibitors

Enzyme inhibitors can be classified as competitive or noncompetitive, and as those binding at the catalytic site, and those binding at an allosteric site (Hille and Massey 1981). Classic competitive inhibitors are substrate analogs, where the inhibitor is structurally similar to the substrate. Allopurinol (4 hydroxypyrazol [3,4-d] pyrimidine) is a substrate analog and competitive inhibitor of xanthine oxidase (Kuzmin *et al* 1995). Hille and Massey (1981) found

that allopurinol is oxidised to oxypurinol (4,6 dihydroxypyrazolo [3,4-d] pyrimidine), which forms a very tight yet reversible binding complex with molybdenum at the active site. Oxypurinol has an extremely high affinity for xanthine oxidase (50% inhibition at 0.5nM) (Parks and Granger 1986a). Its half life in dogs is 4 hours (Lochner *et al* 1990). Other pyrozolo(2,3-d) pyrimidines such as pyrazoloisoguanine and pyrazoloadenine can inhibit xanthine oxidase, but are not as potent as allopurinol and oxypurinol (Parks and Granger 1986a). Folic acid analogs and derivatives, and methylxanthines like theobromine, theophylline and caffeine are also competitive inhibitors of xanthine oxidase (Parks and Granger 1986a). Pterin-6-aldehyde, a photolytic breakdown product of folic acid is probably the active substance which inhibits xanthine oxidase (Parks and Granger 1986a).

Noncompetitive inhibitors bear little or no structural relationship to the substrate, and the inhibition can be reversible or irreversible. Xanthine oxidase is susceptible to inactivation by trace metals, probably due to the formation or exposure of reactive sulphur centres when the substrate reduces the enzyme (Fridovich 1985). Cyanide, arsenite, formaldehyde and methanol are also noncompetitive inhibitors of xanthine oxidase (Parks and Granger 1986a, Roussos 1963). Cyanide and arsenite form complexes with the thiol (sulphydryl) group interfering with the interaction of the enzyme with purines (Parks and Granger 1986a). Methanol is converted to formaldehyde and this donates a proton to molybdenum, inactivating the enzyme (Hille and Massey 1981).

Xanthine oxidase activity is also inhibited by the adrenocortical hormones, cortisone, cortisol and corticosterone (Roussos 1963).

(vi) Enzyme enhancement

Histamine has been shown to enhance the activity of xanthine oxidase from bovine milk (Muraoka 1963), and the liver and intestine of rats (Friedl *et al* 1990, Caty *et al* 1990) although the precise mechanism is unknown. Histamine may enhance xanthine oxidase activity by binding metal ions, such as Cu^{2+}, Pb^{2+} or Ca^{2+}, thereby preventing thier inhibiting effects on the enzyme (Muraoka 1963). However, the amount of histamine liberated during in vivo models of

intestinal ischaemia-reperfusion injury in dogs did not enhance the enzyme's activity *in vivo* (Boros *et al* 1992), so the importance of this interaction is uncertain. Androgens stimulate xanthine oxidase activity (Roussos 1963).

(vii) Half life

There is very little information regarding the half life of xanthine oxidase. Ramboer *et al* (1972) estimated the half life of xanthine oxidase in human blood to be two to three days.

1.4 Ischaemic Disease In Which High Serum Xanthine Oxidase Activity Is A Possible Consequence

When cell membranes are damaged, enzymes may leak into the serum (Ramboer 1972). Some examples are the release of creatine phosphokinase from damaged muscle cells (Hearse *et al* 1973), amylase in pancreatitis, and alanine aminotransferase from damaged hepatocytes (Yokoyama and Parks 1988). Release of xanthine oxidase into the circulation may occur in an analogous manner when endothelial and parenchymal cells are damaged. Circulating xanthine oxidase is increased in humans and rats with viral hepatitis, toxic hepatopathy, hepatic trauma, and in some patients with hepatic neoplasia (Ramboer *et al* 1972, Shamma'a *et al* 1973, Giler *et al* 1975,1976a and 1976b, Yamamoto et al 1996, Batelli *et al* 1996). This is most likely due to release of xanthine oxidase from damaged hepatocytes. *In vitro* release of xanthine oxidase from ischaemia-reperfused rat liver has been demonstrated by Yokoyama and Parks (1988) and Yokoyama *et al* (1990).

Elevation of serum xanthine oxidase activity has been demonstrated following ischaemia-reperfusion injury in species other than dogs. Tan *et al* (1993) found that the serum xanthine oxidase activity was elevated in rats undergoing experimental haemorrhagic shock, followed by reperfusion, and Caty *et al* (1990) demonstrated a rise in circulating xanthine oxidase activity in *in vivo* intestinal ischaemia-reperfusion injury in rats. The xanthine oxidase activity in the serum of a human patient undergoing an aortic cross-clamping procedure (causing ischaemia to the distal half of the body) increased by 80-100% after 7 minutes of reperfusion (release of the clamp) (Tan *et al* 1995). Ischaemia of the distal limb, induced by tourniquet application in rats (Punch *et al* 1992) and humans (Friedl *et al* 1991), followed by reperfusion (tourniquet release) has also been associated with a rise in xanthine oxidase activity in the plasma/serum.

Local ischaemic disorders, particularly of the gastrointestinal tract, may result in injury to distant organs, known as the systemic inflammatory response syndrome (SIRS). The lesions in the distant organs are similar to that seen locally, and are centred around vascular endothelial

damage (Carden *et al* 1993). Acute respiratory distress syndrome (also known as ARDS or "shock lung"), vasculitis, disseminated intravascular coagulation, and multiple organ dysfunction syndrome (MODS) are common sequelae, and death is the usual outcome. Circulating mediators are thought to initiate SIRS (Kirby 1995).

Acute respiratory distress syndrome is characterised by increased pulmonary vascular permeability, leukocyte infiltration and noncardiogenic pulmonary oedema (Carden *et al* 1993). It is generally rapidly progressive over several hours, resulting in the accumulation of highly proteinaceous fluid within the pulmonary parenchyma and airways (Hawkins 1992). ARDS can occur secondary to sepsis, endotoxaemia, parvovirus infection, pancreatitis and disseminated intravascular coagulation (Hawkins 1995). Oxygen derived free radicals (ODFRs) are thought to be involved in the pathogenesis of ARDS, as there is evidence of excessive lipid peroxidation in the lungs (Cross *et al* 1989), and the pathology in ARDS is similar to that seen in pulmonary oxygen toxicity, which is mediated by ODFRs (Jamieson *et al* 1986).

Disseminated intravascular coagulation (DIC) occurs due to the excessive intravascular activation of the coagulation cascade and fibrinolysis mechanism, resulting in microvascular thrombosis and multiple organ failure (Couto and Hammer 1992). Consumption of coagulation factors, platelets, and natural inhibitors of coagulation, such as antithrombin III, lead to paradoxical haemorrhagic and hypercoaguable states (Green and Thomas 1995). DIC is initiated by activation of the extrinsic or intrinsic pathways of the coagulation cascade, or by direct activation of coagulation factors by enzymes released from damaged tissue. This can occur secondary to shock, heat stroke, pancreatitis, hepatic disease, infection and immune mediated haemolytic anaemia (Green and Thomas 1995). Oxygen derived free radicals are known to be directly cytotoxic to the microvascular endothelial cells (Ward *et al* 1986), and therefore could activate the intrinsic pathway, and initiate DIC.

Xanthine oxidase may be a circulating mediator involved in the initiation of ARDS, DIC and SIRS. Xanthine oxidase may react with purine substrates that accumulate in the plasma during ischaemia, and produce large amounts of ODFRs which damage the vascular endothelium (Mousson *et al* 1983, Yokoyama *et al* 1990). Other potential mediators are

tumour necrosis factor (Otto et al 1997), platelet activating factor, thromboxane A2, prostaglandins, leukotrienes (Hawkins 1995), interferon, interleukins, activated complement and procoagulant tissue factors (Kirby 1995).

The serum xanthine oxidase activity in dogs with ischaemic diseases was investigated in the studies reported in this thesis, to determine if increased activity was present in the systemic circulation in a variety of clinical conditions. Such a finding would provide further information regarding the pathogenesis of the systemic complications of ischaemic disease, such as ARDS and DIC. In addition, allopurinol therapy may be beneficial in patients with elevated circulating xanthine oxidase activity.

The ischaemic diseases investigated in the present study included shock (hypovolaemic, septic and endotoxic), gastric dilatation/volvulus, intestinal obstruction, parvovirus (and other causes of haemorrhagic gastroenteritis), pancreatitis, pyometra, heatstroke and immune mediated haemolytic anaemia. The incidence, morbidity and mortality, and pathophysiology of these diseases, and possible mechanisms of xanthine oxidase release into the circulation are discussed below.

(a) Shock (hypovolaemic, endotoxic, septic)

Shock is a condition of progressive circulatory dysfunction, inflicted by exaggerated physiological defences (Michell 1985). Shock can arise due to different etiologies, but leads to a common pathway of poor tissue perfusion (Michell 1985), resulting in the inadequate supply of nutrients and oxygen, and inadequate removal of metabolites from the tissue (Ware 1992). All of the ischaemic diseases that were investigated in this thesis can potentially cause shock.

Hypovolaemic shock results from internal or external loss of fluid eg blood loss, fluid loss in vomit and diarrhoea, and "third spacing" of fluid (Ware 1992). Septic shock (due to the presence of circulating bacteria) and endotoxic shock (due to the presence of circulating endotoxin, a lipopolysaccharide from the cell wall of gram negative bacteria) result in maldistribution of blood flow or volume (Tobias and Schertel 1992). All types of shock result in hypotension, which initiates a number of compensatory mechanisms to maintain the perfusion of "vital" organs - the brain, heart and kidney (Michell 1985). Vasoconstriction of the blood supply to "nonvital" organs results in ischaemia of the liver, gastrointestinal tract and muscle (Ware 1992). If untreated, the animal's condition will deteriorate due to generalised vasodilation, increased capillary permeability (capillaries become "leaky"), depression of cardiac function and worsening hypotension (Michell 1985).

It is apparent that in the early stages shock, ischaemia of the xanthine oxidase-rich liver and intestine occurs, while in advanced stages, a state of total body ischaemia exists (Redl *et al* 1993). Relatively short periods of hypotension will result in liver and intestinal injury - intestinal necrosis occurs within one hour of hypotension (Tobias and Schertel 1992). Administration of intravenous fluids (colloids, crystalloids or blood products), to restore circulating blood volume leads to reperfusion of the ischaemic organs (McCord 1985). Xanthine oxidase, derived from the ischaemic organs, may then enter the circulation via the "leaky" capillaries.

Endotoxic shock, induced experimentally by the intraperitoneal administration of endotoxin in

horses, lead to elevation of serum xanthine oxidase activity (Lochner *et al* 1990), and treatment with allopurinol prior to administration of endotoxin, reduced the severity of the clinical signs associated with shock (Lochner *et al* 1989). Serum xanthine oxidase activity increased in rats with experimental haemorrhagic shock followed by reperfusion by restoration of blood volume (Tan *et al* 1993).

Morbidity and mortality vary according to the severity and type of shock - 50% mortality has been reported in septic shock (Hardie 1992), and Kirby (1995) reported an 80% mortality rate in advanced states of shock. In experimental hypovolaemic shock in the dog and rat, increased survival was demonstrated when animals were pretreated with allopurinol, supporting the role of xanthine oxidase in the pathogenesis of irreversible shock (Crowell *et al* 1969, Badylak 1990).

(b) Gastric dilatation-volvulus

Gastric dilatation-volvulus is the abnormal distension and displacement of the stomach (Berb and Boudrieau 1992). Although gastric dilatation-volvulus is not a particularly common clinical problem in dogs, mortality is often high despite treatment (Leib *et al* 1984). The estimated incidence at the Massey University Veterinary Clinic of approximately 0.13%, is reported to be similar to that at the Veterinary Teaching Hospital of the University of California, Davis (Guilford 1996). Reported mortality rates vary between 25% and 80% (Matthiesen 1985, Wingfield *et al* 1976, Badylak *et al* 1990) with mortality tending to be higher if partial gastrectomy is required to remove necrotic areas from the stomach (Matthiesen 1985). Mortality rates for gastric dilatation without volvulus are lower (10%) (Dann 1976). Chronic gastric volvulus is less common than acute gastric dilatation-volvulus, and the prognosis is better (Willard 1995).

Dilatation and rotation result in increased intraluminal pressure within the stomach, resulting in reduction in the arterial blood flow to the stomach (Boley *et al* 1969), congestion, venous stasis and thrombus formation in the gastric mucosa, and compression of the portal vein and

caudal vena cava (Wingfield *et al* 1976). Obstruction of blood flow to the stomach also occurs due to plugging of the vessels by neutrophils, attempting to migrate into the damaged tissue (Snyder 1989). The stomach becomes ischaemic and oedema, haemorrhage, ulceration and necrosis of the gastric mucosa develops (Lantz *et al* 1984).

Hypovolaemic shock occurs due to pooling of blood in the viscera and "third spacing" of extracellular fluid in the lumen of the gastrointestinal tract, leading to vasoconstriction of the blood supply to the abdominal viscera (Guilford 1996). This, combined with the obstruction of the portal vein (which normally provides venous drainage for the intestine, pancreas and spleen) results in ischaemic damage to these organs (Badylak *et al* 1990). Hepatic congestion develops due to compression of the hepatic veins against the diaphragm, as the liver is pushed cranially by the enlarged stomach (Guilford 1996). Toxins from devitalised tissue and intraluminal bacteria can cross the disrupted gastric and intestinal mucosa, and enter the bloodstream, leading to endotoxic and septic shock (Matthiesen 1985).

Gastric dilatation-volvulus is a classic model of ischaemia reperfusion injury. Many tissues become ischaemic as a direct consequence of the enlarged, malpositioned stomach, and secondary to shock. Reperfusion occurs when the stomach is decompressed and returned to its normal position, and fluids are administered to increase the circulating blood volume. Again, xanthine oxidase could be released into the circulation from the abdominal sites of tissue injury, to lead to SIRS, ARDS and DIC, which are relatively frequent complications of GDV (Wingfield *et al* 1976).

(c) Intestinal obstruction

Acute intestinal obstruction can occur due to foreign bodies, intussusception, intestinal volvulus or strangulated hernias (Burrows *et al* 1995). Obstruction leads to intestinal ischaemia, and hypovolaemic and endotoxic shock (Guilford and Strombeck 1996a). Ischaemic intestinal conditions are associated with high morbidity and mortality (Bulkley *et al* 1981), with a 35%

mortality rate reported in cases of intussusception despite surgical treatment (Weaver 1977).

Obstruction leads to occlusion of venous and lymphatic drainage of the intestine, and in addition, if the obstruction is strangulating, occlusion of the arterial blood supply (Snyder 1989). Mucosal oedema develops secondary to elevated capillary hydrostatic pressure (due to venous obstruction) and the increased capillary permeability, which occurs due to the release of inflammatory mediators from ischaemic tissue (Snyder 1989). Within 4 hours of intestinal ischaemia, cellular infiltration, oedema and haemorrhage will be evident within the mucosa, and the villous epithelium will be separated from the basement membrane (Chiu *et al* 1970, Arden *et al* 1990).

Proximal to the site of obstruction, intraluminal bacteria proliferate and produce exotoxins which stimulate excessive secretion of fluid and electrolytes into the bowel lumen (Burrows *et al* 1995). Sequestration of fluid within the lumen of the gastrointestinal tract, or loss of fluid via emesis lead to hypovolaemic shock. Endotoxins, produced by gram negative bacteria, are able to leak across the highly permeable, damaged ischaemic mucosa, into the systemic circulation where they lead to endotoxaemia and shock (Guilford and Strombeck 1996a).

Thus, in this situation, both the obstruction itself, and hypovolaemic/endotoxic shock, can cause tissue ischaemia. The tissue is reperfused when the foreign body is removed, the intussusception is resected, or the volvulus/strangulation is corrected, and fluids are administered to restore circulating blood volume. Xanthine oxidase may leak into the systemic circulation in a similar manner to the movement of endotoxin.

(d) Parvoviral enteritis and other causes of haemorrhagic gastroenteritis

Canine parvovirus, idiopathic haemorrhagic gastroenteritis, bacterial and other viral infections can cause haemorrhagic diarrhoea, vomiting, dehydration and shock in dogs (Burrows *et al* 1995). Parvovirus is highly contagious and often fatal (Glickman *et al* 1985). Mortality rates ranging from 16% to 50% have been reported (Jacobs *et al* 1980, Black 1979). The virus multiplies within, and destroys intestinal epithelial cells, resulting in increased intestinal permeability and the accumulation of fluid and blood within the bowel lumen (Guilford and Strombeck 1996b). Fatal cases of parvovirus infection frequently show evidence of ARDS (Turk *et al* 1990).

Haemorrhagic gastroenteritis (HGE) is an idiopathic syndrome (Burrows *et al* 1995) which results in ischaemic necrosis and desquamation of the tips of the villi (where xanthine oxidase activity is highest), oedema, vascular stasis and haemorrhage into the lamina propria (Guilford and Strombeck 1996b). The disease is rapidly progressive and death occurs within 24 hours in untreated dogs (Burrows *et al* 1995). The mortality rate is approximately 10% if appropriate fluid therapy is administered (Guilford and Strombeck 1996b).

Infectious organisms causing acute haemorrhagic diarrhoea include *Salmonella, Clostridia, E. coli, Yersinia ,Campylobacter* and coronavirus (Kruth *et al* 1989, Greene 1990, Burrows *et al* 1995). Bacteria may invade the intestinal mucosa, resulting in inflammation and sloughing of mucosa (Greene 1990), and may also secrete exotoxins that increase the secretion of fluid into the lumen of the intestine (Guilford and Strombeck 1996c). Bacteraemia may develop in some situations, particularly in salmonellosis, which can lead to hepatic damage (Guilford and Strombeck 1996c). Coronavirus directly damages the epithelial cells at the tips of the villi (Takeuchi *et al* 1976).

Hypovolaemic shock can develop in all of these situations, secondary to fluid and blood loss in the vomit, diarrhoea and "third spacing" within the lumen of the gastrointestinal tract. Septic and endotoxic shock may also occur after bacteria and endotoxin enter the systemic circulation

across the damaged intestinal mucosa (Guilford and Strombeck 1996c). Dogs infected with parvovirus are particularly susceptible to septicaemia as parvovirus also destroys leukocytes (Burrows *et al* 1995).

Direct injury to the xanthine oxidase-rich intestinal epithelial cells, and, in bacteraemic situations, the xanthine oxidase-rich hepatocytes, and generalised ischaemia secondary to shock, may lead to the release of the enzyme into the circulation, in a similar manner to that described in the situation of intestinal obstruction.

(e)Pancreatitis

Pancreatitis (inflammation, necrosis and oedema of the pancreas gland) is a common diagnosis in canine practice (Richter 1992). The prognosis is guarded (Anderson 1972), and mortality rates of up to 58% have been reported (Schaer 1979).

Digestive pancreatic enzymes are normally stored within zymogen granules to protect the pancreas from self digestion. If the enzymes are activated within the pancreas, inflammatory mediators (such as kallekrein, complement and thromboplastin) are released into the pancreas and blood vessels, causing inflammation, increased pancreatic capillary permeability, decreased pancreatic blood flow and pancreatic ischaemia (Williams 1995). Therefore, conversion of xanthine dehydrogenase to xanthine oxidase could potentially occur in the inflamed, ischaemic pancreas. The pancreas also contains large amounts of the proteolytic enzymes, trypsin and chymotrypsin, which are released from zymogens during pancreatitis (Williams 1995). *In vitro* conversion of xanthine dehydrogenase to oxidase is possible with these enzymes (Batelli 1973). Subsequent entry of xanthine oxidase into the circulation could occur via the "leaky" capillaries.

However, the pancreas contains relatively little xanthine oxidase activity compared with other tissues. Al-Khalidi and Chaglassian (1965) and Schousten *et al* (1983) measured 0.3mU xanthine oxidase per gram of pancreatic tissue, approximately 1% of that found in the intestinal

mucosa. There may be insufficient xanthine oxidase release to elevate serum enzyme activity. But hypovolaemic shock often develops in pancreatitis (Richter 1992) and release of xanthine oxidase from other sites of ischaemia are possible sources of circulating enzyme.

Oxygen derived free radicals have been implicated in the pathogenesis of pancreatitis, with ODFR mediated damage to lysosomes and zymogen granules causing a self perpetuating cycle of tissue ischaemia and destruction (Simpson 1993). Amelioration of the pancreatic oedema, and a reduction in the release of pancreatic amylase from the *ex vivo* canine pancreas in experimentally induced pancreatitis by pretreatment with allopurinol and radical scavengers (catalase and superoxide dismutase), suggests that ODFRs generated by xanthine oxidase play a role (Sanfey *et al* 1985).

Pulmonary oedema and DIC are possible complications of pancreatitis, and may be due to the release of proteases from the inflamed pancreas, which can activate the coagulation cascade and destroy clotting factors (Williams 1995). If serum xanthine oxidase activity is elevated, a possible role in their development must be considered.

(f) Pyometra

Pyometra is a bacterial infection in the uterus, and leads to life threatening bacteraemia and endotoxaemia (Feldman and Nelson 1996). The prevalence is quite low (0.6% Bowen *et al* 1985), but mortality can be up to 11%, even following treatment with intravenous fluids, antibiotics and ovariohysterectomy (Johnson 1995).

Bacterial and endotoxin translocation across the uterine wall and peritoneal cavity may lead to septic or endotoxic shock. Serum xanthine oxidase may rise following fluid therapy (see section 1.4 part a). It is also possible that xanthine oxidase could also be released from the necrotic uterine mucosa, although the uterus contains relatively low xanthine oxidase activity (0.8 mU/gram of tissue) (Al-Khalidi and Chaglassian 1965), approximately 3% of that in the intestinal mucosa.

(g) Heatstroke

Heatstroke is a marked elevation in body temperature (40.5 - 43.0°C) following exposure to high ambient temperature (for example, a dog confined to a car on a hot day) (Lee-Parritz and Pavletic 1992), and is associated with a guarded prognosis (Ruslander 1992).

As body temperature increases the cellular metabolic rate increases, until oxygen supply can no longer meet demands, leading to widespread hypoxia, ischaemia and cell death, particularly in the gastrointestinal tract (Haskins 1995). In addition, dehydration and haemoconcentration lead to sludging of red blood cells in small vessels, predisposing to thrombosis, and causing further ischaemia. It is during this ischaemic phase that xanthine oxidase concentrations may rise within the tissues, to be released during resuscitation with fluid therapy.

DIC is a common complication of heatstroke (Ruslander 1992) and elevated serum xanthine oxidase and ODFRs may play a role in the development of this complication.

(h) Immune-mediated haemolytic anaemia

Immune-mediated haemolytic anaemia is a condition in which the red blood cells are destroyed by the body's immune system, and is associated with a guarded prognosis (Weiser 1985). Rapid haemolysis causes anaemia, resulting in reduced oxygen carrying capacity, tissue hypoxia and ischaemia. This results in similar compensatory mechanisms to those in hypovolaemic shock (Tobias and Schertel 1992), and therefore could result in elevation of serum xanthine oxidase activity in a similar manner to that previously described in shock (see section 1.4 part a)

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Chapter 2

Development of a Spectrophotometric Assay for Measuring Xanthine Oxidase Activity in Canine Serum and the Effect of Storage Temperature and Duration on Xanthine Oxidase Activity

Introduction

Xanthine oxidase activity in serum and tissues can be measured by a variety of methods, including spectrophotometric (Rowe and Wyngaarden 1966, Fridovich 1985), fluorometric (Apple *et al* 1991, Rasanen *et al* 1996), colorimetric (Fried and Fried 1974, Sugiura *et al* 1981), radioisotopic (Dougherty 1975) and manometric techniques (Ball 1939), high performance liquid chromatography (Tan *et al* 1995, Yamamoto *et al* 1996), enzyme linked immunosorbant assay (ELISA) (Price and Harrison 1993), and radioimmunoassay (Bruder *et al* 1983). Enzyme activity is usually assessed by measuring the formation of a product, or the disappearance of a substrate, over time (Roussos 1967). Since xanthine oxidase catalyses the conversion of hypoxanthine and xanthine to uric acid on a mole for mole basis (Roussos 1967), the rate of production of uric acid (Terada *et al* 1990), or the disappearance of hypoxanthine or xanthine (Bray 1962), can be measured to calculate the enzyme activity.

The spectrophotometric method is the most commonly used method (Parks and Granger 1986). A spectrophotometer measures the absorbance of electromagnetic radiation by a solution, which is proportional to the concentration of the analyte in solution. The concentration of the substance is calculated by dividing the absorbance by the molar absorptivity coefficient (Khazanie 1993). The molar absorptivity coefficient for uric acid, at a wavelength of 295 nm, is 1.1×10^4/mol/l/cm (Fridovich 1970). Spectrophotometric methods have been used to measure plasma or serum xanthine oxidase activity in humans (Johnson and Rajagopalan 1976, Ramboer 1969, Ramboer *et al* 1972, Majkic-Singh *et al* 1987), and rats (Fried *et al* 1990), but not in dogs. The purpose of this study was to develop a spectrophotometric assay of uric acid production, for measuring the xanthine oxidase activity in canine serum.

Establishment of any new laboratory assay requires the assessment of the linearity, technical inaccuracy, sensitivity, reproducibility, analysis time, cost and clinical usefulness of the assay (Hartmann 1992). These qualities of the spectrophotometric assay were investigated in this study. Linearity is defined as the range of an analyte concentration over which the assay is linear or usable. Inaccuracy is defined as the difference between the measurement made by the assay, and the true value of the sample. Reproducibility is defined as the agreement between replicate measurements, and is described in terms of a coefficient of variation. Reproducibility should be evaluated within runs (intra-assay variation) and between different days of assay (inter-assay variation) (Hartmann 1992).

For convenience, sometimes specimens need to be stored for a period of time prior to assay (Parks *et al* 1988). The effect of storage duration and temperature on the activity of xanthine oxidase in canine serum has not been investigated to date. Rat serum xanthine oxidase activity is stable for up to 11 days when stored at 4°C (Ramboer 1969), and equine serum xanthine oxidase activity is known to be stable at -70°C for 4 days (Lochner *et al* 1990). Bovine milk xanthine oxidase activity is known to be stable for 30 days when stored at -20°C (Dougherty 1976, Fried and Fried 1974), but when stored in human albumin at 4°C, a slow decline in activity is seen, with a half life of 35 days (Ramboer 1969).

Commercially purified bovine milk xanthine oxidase is an accessible source of this enzyme (Price and Harrison 1993). The kinetics of xanthine oxidase derived from different sources appear to be similar (McCarthy and Long 1976). Since canine serum contains relatively small amounts of xanthine oxidase activity (44 mU/l Al-Khalidi and Chaglassian 1965), it was 'spiked' with bovine milk xanthine oxidase, to create suitable specimens for the assessment of the properties of the spectrophotometric assay, and the effect of storage duration and temperature.

Materials and Methods

Materials

Xanthine (2,6-dihydroxypurine), oxonate (allantoxanic acid as a potassium salt), and purified bovine milk xanthine oxidase (containing 0.57 Units of Xanthine Oxidase per milligram (mg) of protein, 28 milligrams of protein per millilitre (ml), and therefore 15.96 Units/ml), were obtained from the Sigma Chemical Company, St Louis, USA. Sodium dihydrogen orthophosphate 1-hydrate ($NaH_2PO_4.H_2O$) and di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4), were obtained from BDH Laboratory Supplies, Poole, England ("AnalaR®").

Solutions

Solutions were freshly prepared on each day of assay.

(1) Phosphate Buffer Solution 0.1 mol/l, pH 7.4

317 mg of sodium dihydrogen orthophosphate-1hydrate and 1093 mg of di-sodium hydrogen orthophosphate anhydrous were dissolved in 100 ml of distilled water. The pH was measured using a pH meter and was adjusted to 7.4 by addition of 0.1 mol/l sodium dihydrogen orthophosphate, or 0.1 mol/l di-sodium hydrogen orthophosphate.

(2) Xanthine (substrate) 1 mmol/l

15.2 mg of xanthine was dissolved in 0.2 ml of 1 mol/l sodium hydroxide, then distilled water was added to a total volume of 100 ml.

(3) Oxonate 1.5 mmol/l

29.3 mg of oxonate was dissolved in 100 ml of distilled water.

(4) Bovine milk xanthine oxidase was diluted in 0.1 mol/l phosphate buffer solution (see *experimental design* section for ratios used).

Serum

Blood was collected from the jugular veins of four dogs, using a 22 gauge needle and syringe. The blood was placed in a plain sterile vacutainer, allowed to clot, centrifuged at 3000 rpm for 5 minutes, and the serum supernatant was collected. Serum was pooled and stored at -80°C until required. Varying amounts of bovine milk xanthine oxidase was added to serum in each

experiment (detailed below). Collection of blood was approved by the Massey University Animal Ethics Committee.

Procedure for the assay

The following volumes of each reagent were placed in a 10 ml glass test tube:

1.5 ml of 0.1 mol/l phosphate buffer

0.3 ml of 1 mmol/l xanthine

0.2 ml of 1.5 mmol/l oxonate

0.9 ml of distilled water

The reaction was commenced by the addition of 0.1 ml of serum (containing bovine milk xanthine oxidase). The total volume of the reaction mixture was 3 ml. Immediately following the addition of serum, the mixture was agitated for 3 seconds using a Chiltern mixer.

The spectrophotometer (Philips SP8-400, with a deuterium lamp lightsource) was zeroed with distilled water. Using a pipette, an aliquot of the reaction mixture was transferred to a Quartz cuvette with a 1 cm light path, and the absorbance was measured at 295 nm. The aliquot was then transferred back to the test tube until the time of the next absorbance measurement. The test tube was agitated for 3 seconds before and after each measurement of absorbance to ensure adequate mixing. The cuvettes were cleaned between measurements with lavages of distilled water, and air-dried. The assay was performed at ambient temperature (approximately 20-25°C).

Calculation

One International Unit of xanthine oxidase activity can be defined as the amount which produces 1µmol of uric acid per minute (Worthington 1988). The amount of uric acid produced, and hence the xanthine oxidase activity, can be calculated by using the change in absorbance per minute:

$$\text{Change in absorbance /minute } (\Delta A/\text{min}) = \frac{\Delta \text{ Absorbance in n minutes}}{n}$$

$$\text{mmol uric acid/minute/0.1 ml serum} = \frac{\Delta A/\text{min} \times 3 \text{ ml reaction volume}}{\text{molar absorptivity coefficient}}$$

$$\text{mmol uric acid per minute/ml serum} = \frac{\Delta A/\text{min} \times 3 \text{ mls}}{1.1 \times 10^4 \times 0.1 \text{ ml serum}}$$

$$\text{µmol uric acid per minute/ml serum} = \frac{\Delta \text{Absorbance/minute} \times 3 \text{ mls} \times 10^3}{1.1 \times 10^4 \times 0.1}$$

$$\text{(simplified to)} = \Delta \text{Absorbance/minute} \times 2.727$$

Therefore:

$$\text{Units XO/litre serum} = \text{µmol uric acid/min/litre} = \Delta \text{Absorbance/minute} \times 2.727 \times 10^3$$

Experimental design

(1) Determination of the Kinetics, Linearity and Accuracy of the assay

Twenty microlitres (µl) of a 1:10 dilution of xanthine oxidase was added to the reaction mixture containing 0.1 millilitres (ml) of canine serum, and assayed as described above. The absorbance was measured at two to five minute intervals for 65 minutes, to assess enzyme kinetics.

To determine the linearity, varying amounts of a bovine milk xanthine oxidase were added to test tubes containing the reaction mixture (0, 0.13, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 milliunits, respectively), and the change of absorbance was measured between 30 and 60 minutes. To determine the accuracy of the assay, the measured xanthine oxidase activity was compared with

the expected result, based on the enzyme activity of purified bovine milk xanthine oxidase.

(2) Assessment of the effect of the addition of canine serum to the assay

Variable amounts of canine serum was added (0, 10, 20, 60 and 100 µl respectively) to reaction mixtures containing 50 µl of a 1:100 dilution bovine milk xanthine oxidase. The change in absorbance was measured every 5 to 10 minutes over a 60 minute period, and the reaction rates were compared.

(3) Assessment of the effect of the addition of oxonate to the assay

To assess the effect of the addition of the uricase inhibitor oxonate, 50µl of a 1:100 dilution of bovine milk xanthine oxidase, and 0.1 ml of canine serum were added to test tubes containing the reaction mixture with or without the oxonate solution. The absorbance was measured every minute for 10 minutes, and the reaction rates were compared.

(4) Determination of inter-assay and intra-assay variation

To create specimens with high and moderate xanthine oxidase activity, 100 µl and 25 µl of a 1:10 dilution of bovine milk xanthine oxidase, was added to two 2 ml serum samples, respectively. The serum was agitated for 3 seconds using a Chiltern mixer, and immediately assayed for xanthine oxidase activity, as described above. Assays were repeated six times for each sample (intra-assay variation). The remaining serum containing xanthine oxidase, was divided into twelve plastic serum storage vials, and stored at -80°C for up to 90 days. On 6 different days, one vial each of serum containing high and moderate xanthine oxidase activity, was thawed at room temperature, and assayed for xanthine oxidase activity.

(5) Determination of the effect of storage temperature and duration on bovine milk xanthine oxidase stored in canine serum

Three hundred and seventy five µl of a 1:10 dilution of bovine milk xanthine oxidase was added to 15 millilitres of serum. The serum was agitated for 3 seconds using a Chiltern mixer, and then divided equally into twenty plastic serum storage vials. Serum samples were stored for up to 90 days at +4°C (refrigerator), -20°C (standard freezer), and -80°C (biofreezer). Samples were thawed at room temperature. The xanthine oxidase activity was measured after zero, 1, 5, 7,

14, 30 and 90 days of storage, using the assay described above. Measurements on each sample were repeated five to six times.

Statistics

Intra-assay and inter-assay variation and Storage Study: The mean xanthine oxidase activity, standard deviation , and coefficient of variation were calculated using standard statistical methods (Strike 1991). The coefficient of variation (CV), expressed as a percentage, was calculated by the following formula: $CV = \frac{\text{Standard deviation}}{\text{mean}} \times 100\%$

Results

(1) Kinetics, Linearity and Accuracy

The reaction kinetics (change in absorbance over time) is shown in Figure 2.1. After a short "burst phase" of approximately 8 minutes duration, the formation of uric acid by xanthine oxidase, appears to follow zero order kinetics.

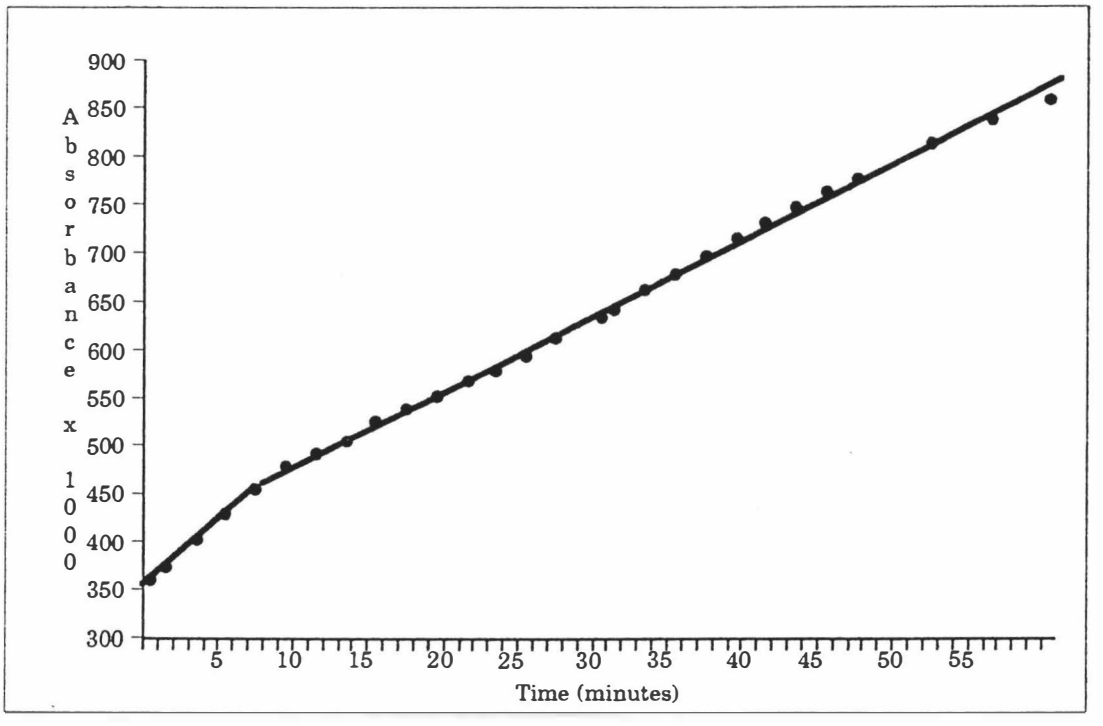

Figure 2.1 Enzyme kinetics of Bovine Milk Xanthine Oxidase in Canine Serum

Figure 2.2 shows that the change in absorbance measured is proportional to the amount of xanthine oxidase, and confirms linearity up to 80 Units/l (which is equivalent to the addition of 8 mU of bovine milk xanthine oxidase to the reaction mixture). Table 2.1 shows the amount of xanthine oxidase activity, measured using the spectrophotometric assay, in each specimen, compared with the amount of activity expected to be measured, based on the volume of bovine milk xanthine oxidase added to the serum. At low xanthine oxidase activities the assay appears accurate (less than 2.5 U/l), but at higher activities, the accuracy falls, so that approximately one half of the actual activity is measured.

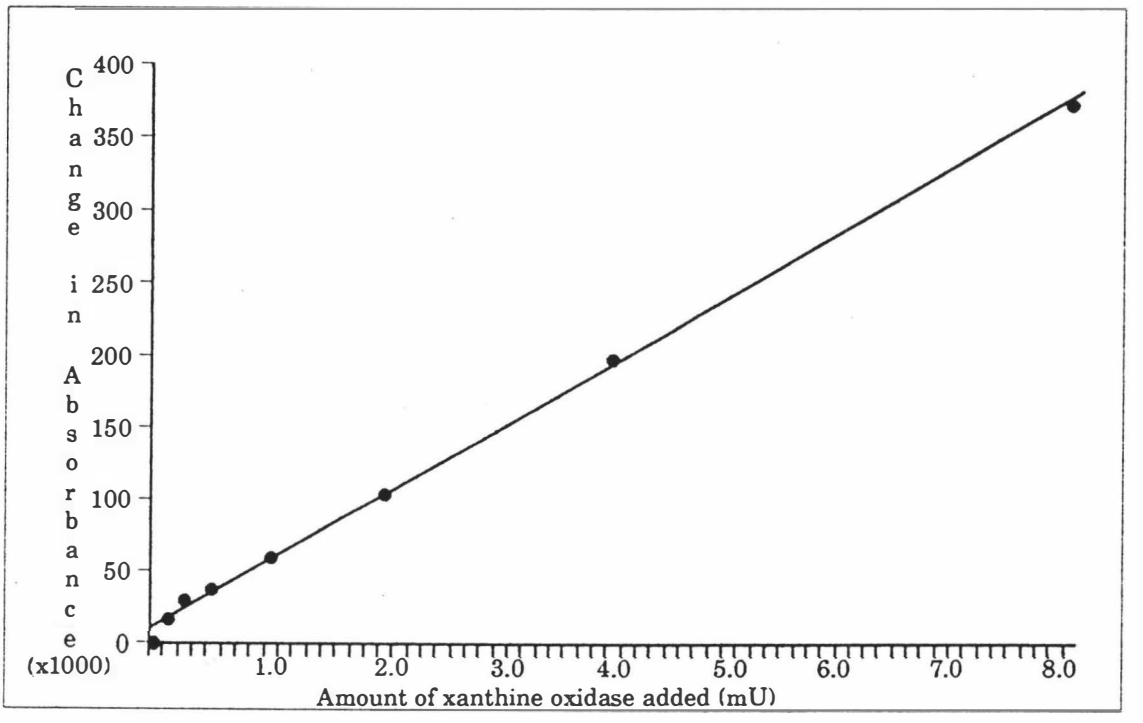

Figure 2.2 The Change in Absorbance (Reaction Rate) at Varying Xanthine Oxidase Activities

Actual Xanthine Oxidase U/l	Measured Xanthine Oxidase U/l
1.3	1.5
2.5	2.6
5	3.4
10	5.4
20	9.5
40	18.1
80	39.1

Table 2.1 Measured and Actual Xanthine Oxidase Activities

(2) Effect of the Addition of Canine Serum

The change in absorbance over time (reaction rates), for reaction mixtures containing varying amounts of xanthine oxidase, is shown in Figure 2.3. The addition of canine serum increased the initial absorbance measured, but the change in absorbance over time remained unchanged. Therefore, the addition of canine serum does not interfere with the assay.

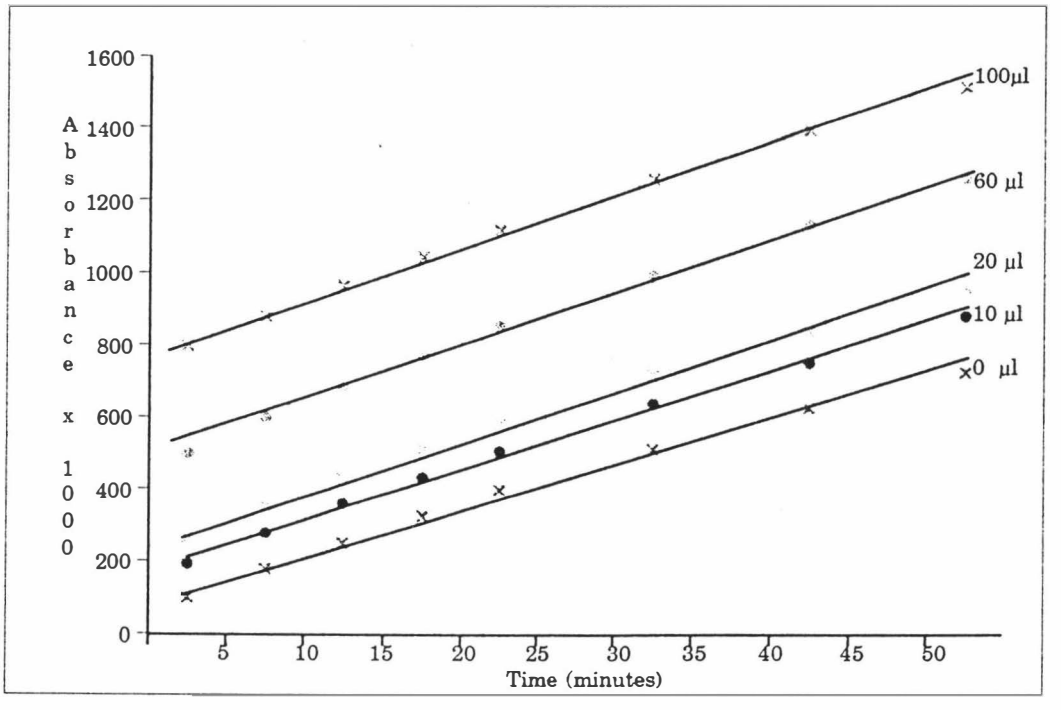

Figure 2.3 The Effect of Varying Quantities of Canine Serum on the Reaction Rate (Change in Absorbance)

(3) Effect of the Addition of Oxonate

The addition of oxonate to the reaction mixture resulted in a small increase in the rate of the reaction (change in absorbance) (Figure 2.4). Oxonate does not interfere with the assay.

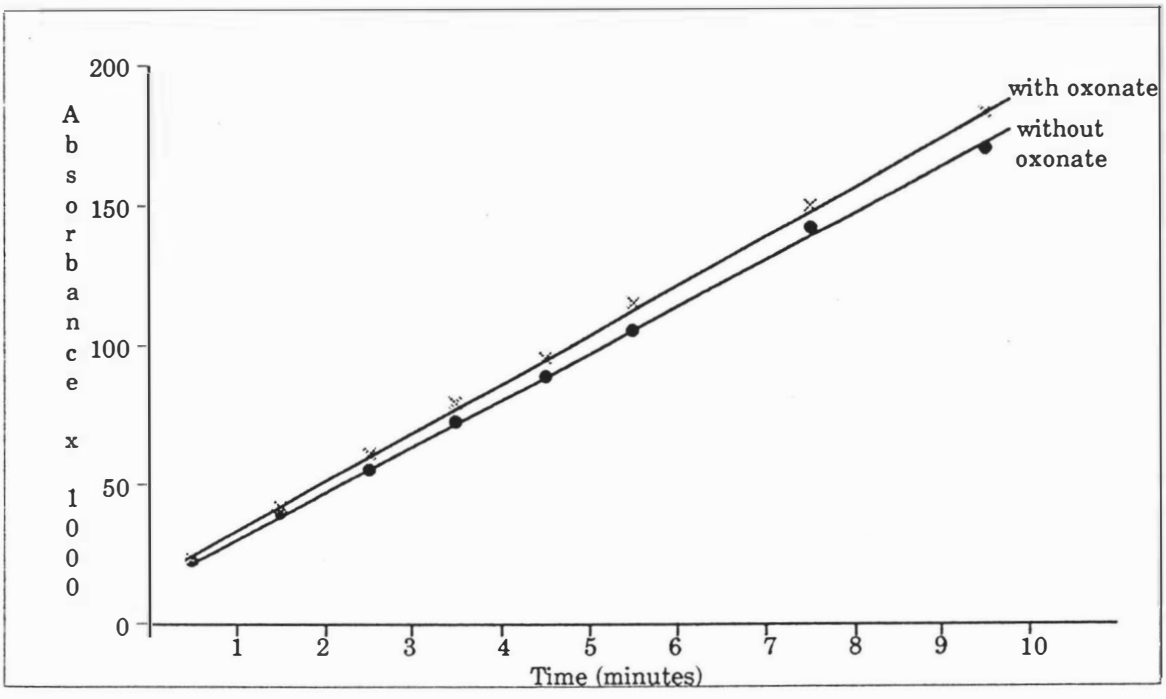

Figure 2.4 Reaction Rates, With or Without Oxonate

(4) *Intra-assay and Inter-assay variation*

Data from the intra-assay and inter-assay variation experiments are shown in Tables 2.2 and 2.3, respectively. For specimens containing high xanthine oxidase activity, the intra-assay and inter-assay coefficients of variation were 2.1% and 10.7% , respectively. For specimens containing moderate xanthine oxidase activity, the intra-assay and inter-assay coefficients of variation were 5.5% and 12.8% , respectively.

Xanthine Oxidase U/l	Mean	SD	CV
High XO specimens			
34.7			
35.4			
33.8			
34.4			
33.6			
33.5	34.2	0.7	2.1%
Moderate XO specimens			
13.2			
12.5			
12.6			
12.7			
11.7			
11.4	12.4	0.7	5.5%

Table 2.2 Intra-assay Variation Data for Specimens Containing High and Moderate Xanthine Oxidase Activity. SD = Standard Deviation, CV = Coefficient of Variation

Xanthine Oxidase U/l	Mean	SD	CV
High XO specimens			
33.8			
34.4			
32.3			
31.5			
27.0			
26.8	31.0	3.3	10.7%
Moderate XO specimens			
12.7			
12.0			
15.4			
14.7			
16.9			
15.8	14.6	1.9	12.8%

Table 2.3 Inter-assay Variation Data for Specimens Containing High and Moderate Xanthine Oxidase Activity. SD = Standard Deviation, CV = Coefficient of Variation

(5) Storage Study

The mean ± the standard deviation of xanthine oxidase activities measured in serum stored at +4°C, -20°C and -80°C for up to 90 days are shown in Table 2.4. There appeared to be a trend towards an increase in serum xanthine oxidase activity, at all temperatures studied, over the first 7 days of storage. The increase was mild (3%) in specimens stored at +4°C, but larger at colder temperatures (17% and 20% increase at -20°C and -80°C, respectively). Xanthine oxidase activity appeared stable in serum stored at +4°C for 30 days, but declined by 17% after 90 days of storage. It was noted that the serum became very viscous and was difficult to pipette at this stage. After the initial rise in enzyme activity, xanthine oxidase activity appeared stable when stored at -20°C and -80°C for the 90 days period studied (see Figure 2.5).

Temperature	Number Of Days Stored						
of storage	0	1	5	7	14	30	90
+4°C	20.64 ± 0.99	19.34 ± 0.97	19.90 ± 0.46	21.30 ± 0.37	21.18 ± 0.58	21.45 ± 0.94	17.49 ± 1.22
-20°C	20.64 ± 0.99	20.34 ± 0.54	22.24 ± 0.95	24.01 ± 0.51	21.83 ± 0.73	24.04 ± 0.35	22.70 ± 1.71
-80°C	20.64 ± 0.99	20.34 ± 0.52	22.48 ± 0.68	24.80 ± 0.70	22.87 ± 0.67	26.63 ± 0.74	26.93 ± 1.36

Table 2.4 Mean Xanthine Oxidase Activity ± Standard deviation

After Storage at +4, -20 and -80°C For Up To 90 Days

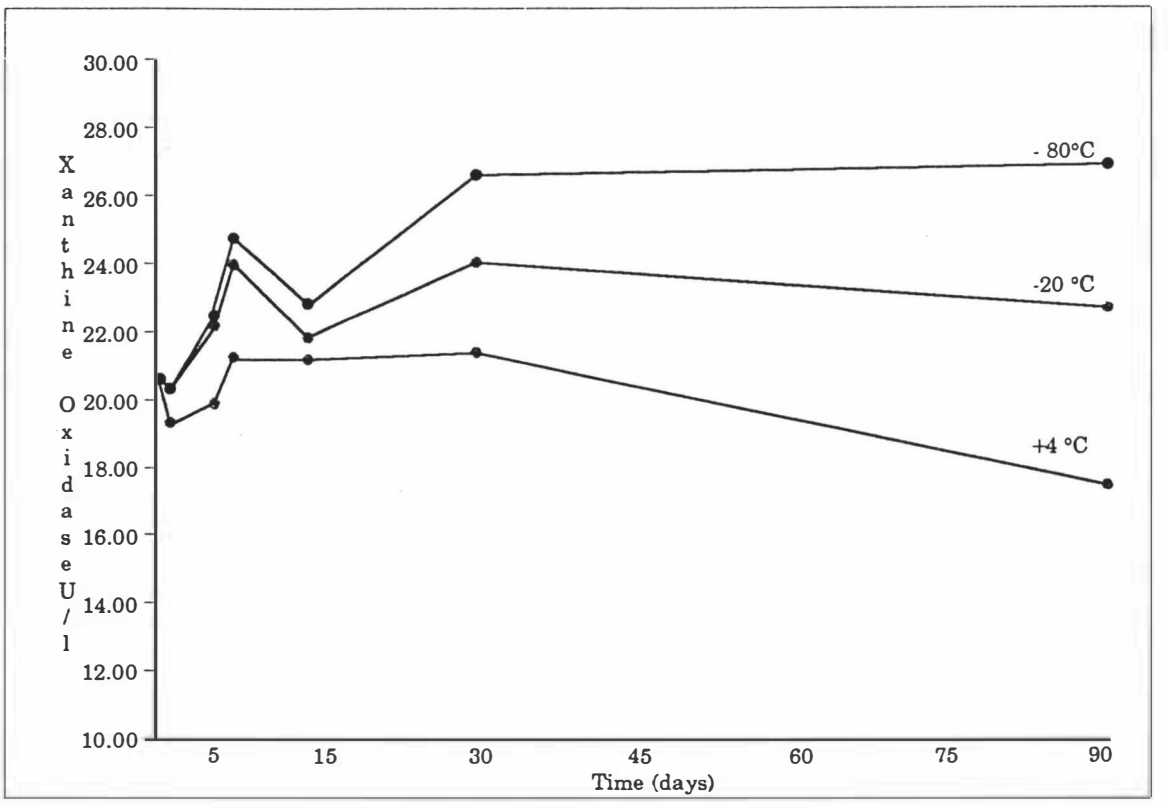

Figure 2.5 The Effect of Storage Temperature and Duration on the Activity of Bovine Milk Xanthine Oxidase Stored in Canine Serum

Discussion

The rates of enzyme catalysed reactions are affected by pH, temperature, and substrate concentration (Rodwell 1988). These factors have not been standardised in the various methods of determination of xanthine oxidase activity. This leads to difficulty in comparing enzyme activities measured in different studies. Even the definition of a unit of xanthine oxidase is inconsistent. It is generally agreed that one unit of xanthine oxidase is that amount which catalyses the production of 1 μmol of uric acid from xanthine or hypoxanthine, per minute (Stirpe and Della Corte 1969), but the temperature of the assays has ranged from 25°C (Worthington 1988) to 37°C (McKelvey et al 1988) , and the pH has ranged from 7.5 (Worthington 1988) to 8.3 (Rowe and Wyngaarden 1966, Ramboer *et al* 1972).

The optimum pH for xanthine oxidase activity varies according to source of the enzyme and the type of buffer used. Some examples are shown in Table 2.5. Similarly, the optimum temperature for xanthine oxidase varies between tissue types. For example, the optimum temperature for xanthine oxidase activity in equine serum, bovine milk and bovine small intestine is 28°C (Lochner *et al* 1990), 37°C (Fried 1966), and 47-50°C (Roussos 1967), respectively. The latter temperature is surprising given that protein denaturation would be expected to occur at such high, unphysiological temperatures (Rodwell 1988).

Tissue type	Optimum pH	Reference
Equine serum	7.8	Lochner et al 1990
Bovine milk	8.6	Ramboer 1969
Rat Liver	8.35	Ramboer 1969
Human liver	7.0	Wajner and Harkness 1989
Human cerebrum	7.5	Wajner and Harkness 1989
Bovine intestine:		
Potassium Phosphate Buffer	7.5 - 7.7	}
Glycine Buffer	8.6 - 8.8	} Roussos 1963
Tris Chloride Buffer	8.3 - 8.7	}

Table 2.5 Optimum pH for Xanthine Oxidase Activity in Various Tissues and in the Presence of Various Buffer Types

No attempt was made in this study to determine the optimum pH and temperature for the activity of bovine milk xanthine oxidase in canine serum. Instead, it was decided to select an appropriate pH and temperature, and maintain consistency throughout the assays. A reaction pH of 7.8 was chosen, as this is the optimum pH for equine serum xanthine oxidase (Lochner *et al* 1990), and the pH most commonly used in the definition of a unit of xanthine oxidase. The final pH of the reaction mixture varied between 7.6 and 7.8. A temperature of approximately 20°C to 25°C (ambient temperature) was chosen for convenience.

Substrate concentrations ranging from 1.5 μmol/l (Al-Khalidi and Chaglassian 1965) to 5 mmol/l (Fried *et al* 1990), have been used in assays of xanthine oxidase activity, with 50-100μmol/l used most commonly. The Km for xanthine, at 25°C and pH 7.8, is approximately 1 μmol/l (Fridovich 1985), and at substrate concentrations much greater than Km, the reaction rate is maximal (Rodwell 1988), which is desirable in this assay. However, substrate inhibition occurs at concentrations greater than 2 mmol/l of xanthine (Bray 1962). Fried and Fried (1974) reported an optimum substrate concentration of 100-200 μmol/l xanthine, and thus a substrate concentration of 100 μmol/l was chosen for this assay.

A burst phase of product formation, followed by a slower rate of reaction (steady state phase), is a typical occurrence in enzyme catalysed reactions (Rodwell 1988). When an enzyme catalyses a chemical reaction, the enzyme molecules undergo a physical change, and become temporarily inactive. As the reaction is completed, the molecules revert to their active form. The burst phase corresponds with the conversion of all of the enzyme molecules to the inactive form, and the steady state phase occurs as active enzyme molecules are regenerated (Rodwell 1988). Following the burst phase, the rate of uric acid production is constant (the reaction follows zero order kinetics), which is in agreement with the enzyme kinetics demonstrated in human serum by Ramboer (1969), and Giler *et al* (1975), and in the tissues of humans and laboratory animals (Terada *et al* 1990, Wajner and Harkness 1989, Majkic-Singh *et al* 1987).

The amount of xanthine oxidase activity measured by the assay was less than the expected amount of activity, based on the stated activity of the commercially prepared bovine milk xanthine oxidase. This is most likely a reflection of the different conditions under which the

assays were performed. The xanthine oxidase activity of the bovine milk product used in this study is stated to be approximately 15.96 units per ml, where one unit converts 1 μmol of xanthine to uric acid per minute, at a pH of 7.5 and temperature of 25°C. Even relatively small changes in pH and temperature can markedly affect an enzyme's activity. For example, the activity of xanthine oxidase in a specimen of equine serum at a pH of 7.8 was 60 mU/l , but fell to 35 mU/l at a pH of 7.4, and the activity fell from 55 mU/l to 36 mU/l when the temperature of reaction was changed from 28°C to 20°C (Lochner *et al* 1990).

Another possible explanation for the apparent inaccuracy seen is the presence of inhibitors, such as trace metals (which may contaminate buffer salts), or uric acid. Some authors have included a chelating agent such as EDTA, to bind trace metals (Fridovich 1985). In one experiment, the addition of EDTA to the reaction mixture increased the milk xanthine oxidase activity fourfold, but did not affect the activity of the enzyme from liver or serum (Ramboer 1969). Uric acid can inhibit xanthine oxidase at high concentrations, but does not affect the enzyme below 0.9 mmol/l (Yamamoto *et al* 1996). If the concentration of uric acid in canine serum is very low, the xanthine oxidase activity would have to exceed 450U/litre serum before the concentration of uric acid in the reaction mixture exceeded 0.9mmol/l. The xanthine oxidase activity expected to be measured in dogs is likely to be measured in milliunits per litre (Al-Khalidi and Chaglassian 1965), therefore, inhibition by uric acid is unlikely to be significant.

The presence of canine serum in the reaction mixture does not interfere with the assay, but does increase the initial absorbance. The high absorbance of canine serum is due to the plasma proteins, rather than naturally occurring high quantities of uric acid, as dialysis to remove the uric acid does not significantly reduce the absorbance (M Wiseman, personal communication). The addition of 100μl of canine serum resulted in an initial absorbance of approximately 0.8. The maximum absorbance able to be measured by the spectrophotometer is 3.0. Therefore, more than 100 μl of serum could be added to the reaction mixture to increase the sensitivity of the assay. Up to 400 μl of serum has been used in assays of equine xanthine oxidase activity (Lochner *et al* 1990).

Uricase is an enzyme that degrades uric acid, and measurement of uric acid production by this

assay would obviously be hampered if significant uricase activity was present in the serum (Beckman *et al* 1989). Addition of the uricase inhibitor (oxonate) to the reaction mixture may not be necessary when measuring the activity of canine serum, as the reaction rate observed in this study was similar with or without oxonate. However, the uricase activity in canine serum is likely to be variable, and since oxonate does not interfere with the assay, it was added as a precaution.

There will always be a certain amount of variability in repeated measurements, which is affected by the operator technique, the procedure of the assay, and the instrument used (Bakes-Martin 1993). A coefficient of variation less than 5% is desirable (Bakes-Martin 1993). This level of precision was achieved within batches of assays (intra-assay variation), but the coefficient of variation was slightly higher in samples assayed on separate days. Possible sources of variation include fluctuations in environmental temperature, small differences in the preparation of buffers and solutions, the volume of solution delivered by pipettes, and the accuracy of the spectrophotometer in measuring absorbance.

From the storage study data, it appears serum can be stored for less than 30 days at 4 °C, -20°C or -80 °C without a decrease in xanthine oxidase activity,but for longer periods of storage, a temperature of --20 °C or -80 °C is necessary. The increase in the activity of bovine milk xanthine oxidase stored in canine serum, over the first 7 days of storage, was an interesting finding. This may represent a true increase in enzyme activity, or be merely a reflection of inter-assay variation. Storage of some tissues can cause an increase in xanthine oxidase activity. Storage of liver (from rabbits, rats, man), at 4°C and -20°C, can result in up to an 8 fold increase in the xanthine oxidase activity over a 1 to 2 week period, followed by a slow decline in activity (Wajner and Harkness 1989, Ramboer 1969). Storage at 4°C resulted in a larger increase than at -20°C (Ramboer 1969). The increase in activity is thought to be due to ongoing conversion of xanthine dehydrogenase to xanthine oxidase in the tissues during storage (Stirpe and Della Corte 1969, Wajner and Harkness 1989). The dehydrogenase activity in canine serum has not been measured, but Yamamoto *et al* (1996), found that the xanthine dehydrogenase activity in human plasma was negligible, so ongoing conversion may not be the reason for the increased activity seen. Other possible explanations are that endogenous

inhibitors may disappear during storage (Ramboer 1969), or that evaporation of water from the serum during storage increased the concentration of the enzyme in the serum.

The spectrophotometric method is simple, practical and cheap, with materials costing only a few cents per test. While other methods, such as the fluorometric, radioisotopic, ELISA, and radioimmunoassay techniques are more sensitive (Beckman *et al* 1989, Dougherty 1976, Price and Harrison 1993, Jarasch *et al* 1986), this method has the advantages that it is less time consuming, and less costly than some of the alternative methods, and does not require the storage or handling of radioactive substances. The spectrophotometric method described above should have adequate sensitivity for the purpose of measuring elevated xanthine oxidase activity in canine serum, particularly if larger aliquots of serum are used. It can be calculated that the assay can detect as little as 45 mU/l using 100µl aliquots of canine serum, but if 400µl aliquots of serum are used, as little as 11 mU/l can be detected. Al-Khalidi and Chaglassian (1965) reported a mean serum xanthine oxidase activity of 44 mU/l in dogs, therefore it is expected that by assaying 200 to 400µl aliquots of serum, "healthy" dogs will have no, or barely detectable xanthine oxidase activity, but any elevation in serum xanthine oxidase activity that might occur in ill dogs will be detectable. The serum xanthine oxidase activity in ill dogs has not been investigated, but in experimental endotoxic shock in horses, the serum xanthine oxidase increased to 500 - 3000 mU/l (Lochner *et al* 1990). If similar increases occur in dogs undergoing ischaemia-reperfusion injury, this will be easily detected.

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Chapter 3

Serum Xanthine Oxidase Activity in "Healthy" Dogs and the Estimation of a Reference Interval

Introduction

The development and verification of any new analytical method requires the evaluation of reference values from a sample population, and the construction of a reference interval (Hartmann 1992). Clinical pathology from a patient is meaningless unless it can be compared with reference information (Dybkær and Solberg 1987), which serves as a type of control for the patient under study (Grasbeck 1990).

The terminology and methodology relating to the concept of reference values remained controversial until 1970, when the International Federation of Clinical Chemistry (IFCC) formed a Committee to standardise nomenclature and recommend procedures for the production of reference values and their statistical treatment (Solberg 1987b, Solberg and Grasbeck 1989). Use of ambiguous terminology such as "normal values" and "reference range" is now discouraged. The word "normal" has conflicting meanings in different contexts. In a clinical context, "normal" means nonpathological or healthy (Grasbeck 1990), but in a statistical context, "normal" implies that data fits a Gaussian (bell shaped) frequency distribution curve (Solberg and Grasbeck 1989). However, the activity of serum enzymes in clinically "normal" individuals does not usually fit a Gaussian Distribution (Bush 1991, Wiedemann *et al* 1993). The range is the difference between the highest and lowest values in a data set, and is a single numerical value (Bakes-Martin 1993), rather than a pair of numbers which frequently are said to represent the "reference range". Currently the reference interval is the preferred terminology (Solberg 1987a).

Other definitions of nomenclature provided by the IFCC, relating to reference values include: (1) reference value- the value obtained by measurement of a particular type of quantity on an

individual belonging to a reference sample group

(2) reference sample group - a number of reference individuals, taken to represent the reference population (which is a hypothetical entity and consists of all possible reference individuals)

(3) reference distribution - the statistical distribution of the reference values.

(4) reference limit - a value derived from the reference distribution, used for descriptive purposes. A reference limit is usually defined so that a stated percentage of the reference values would be less than or equal to the limit with a stated probability. Therefore the reference interval is the interval between and including the two reference limits (Solberg 1987a).

Many methods described for the determination of reference intervals are misused, as they are parametric, and therefore are only applicable if the data assumes a Gaussian distribution. A common example of a parametric method is the use of the mean ± 1.96 standard deviations to define the limits containing 95% of the population (Bermes *et al* 1976). Since reference data pertaining to serum enzyme activities does not usually fit such a distribution (Bakes-Martin 1993), non-parametric methods have been described (Reed *et al* 1971) and are recommended by the IFCC (Solberg 1987b).

The serum xanthine oxidase activity in "healthy" dogs has not been thoroughly evaluated. In 1965, Al Khalidi and Chaglassian measured the serum xanthine oxidase activity in three dogs using a radioisotopic assay. The purpose of this investigation was to measure the serum xanthine oxidase activity in a sample of clinically "healthy" dogs, and to determine the reference distribution, reference limits and a reference interval, using the recommendations of the IFCC.

Materials and Methods

Animals

Thirty-nine dogs (25 female, 14 male) with ages ranging from 4 months of age to 13 years were used as reference individuals. A variety of breeds were represented (New Zealand working dog n=13, German Shepherd n=6, Labrador n=3, Doberman n=3, Huntaway, Poodle, Border Collie, Fox terrier each n=2, and German Shorthaired Pointer, Daschund, Jack Russell Terrier, Golden Retriever, Elkhound and Crossbred dog, each n=1). Sources of reference individuals included dogs presented to the Massey University Veterinary Clinic for vaccination or routine elective surgery (ovariohysterectomy or castration), dogs owned by staff or students of the Massey University Veterinary Clinic, or dogs bred by the Animal Health Services Centre of Massey University. Dogs were considered to be healthy on the basis of a physical examination and absence of history of illness in the preceding 60 days. Owner consent was obtained prior to the collection of blood samples and all procedures were approved by the Massey University Animal Ethics Committee.

Serum

One to 3 millilitres of blood was collected from the cephalic or jugular vein of each dog, with a 20 or 22 gauge needle and 3 ml syringe. The blood was placed into a plain sterile vacutainer (Becton Dickinson Vacutainer Systems, Franklin Lakes, New Jersey, USA) and allowed to clot at room temperature (approximately 20°C). The vacutainer and its contents were then centrifuged at 3000 rpm for 5 minutes, then the serum supernatant was aspirated and stored in plastic storage vials at -80°C for less than 90 days. Prior to assay, the serum was thawed at room temperature.

Materials

Xanthine and oxonic acid were obtained from the Sigma Chemical Company, St Louis, Missouri, USA, and analytical grade sodium dihydrogen orthophosphate1-hydrate and di-sodium hydrogen orthophosphate anhydrous were obtained from BDH Laboratory Supplies, Poole, England. Solutions were prepared as described in Chapter two.

Serum xanthine oxidase assay

Serum xanthine oxidase activity was measured using the spectrophotometric method described in Chapter two. The method was modified by the addition of larger volumes of serum (0.2 or 0.4 ml of serum rather than 0.1 ml), and the amount of distilled water added to the reaction mixture was reduced (0.8 or 0.6 ml respectively, rather than 0.9 ml), to maintain a total volume of 3 ml.

Calculation of serum xanthine oxidase activity

The formula described in Chapter two was modified for the addition of variable amounts of serum:

$$\text{Units xanthine oxidase (XO) /ml serum} = \frac{\Delta\text{Absorbance (in 60 minutes)} \times 10^3 \times 3}{60 \times 1.1 \times 10^4 \times \text{volume of serum (ml)}}$$

which is simplified to:

$$\text{U XO/ml} = \frac{\Delta\text{Absorbance in 60 minutes}}{220 \times \text{volume of serum (ml)}}$$

The number of units of xanthine oxidase per millilitre was multiplied by 10^6 to convert them to milliUnits per litre (mU/l).

Statistics

The mean and range of the reference values for serum xanthine oxidase activity were calculated using standard statistical methods (Sachs 1984). The Kruskal Wallis One Way Analysis of Variance test was used to determine if there was a significant difference between the reference values from male and female dogs. The Pearson Correlation Coefficient was calculated to examine the relationship between age and serum xanthine oxidase activity.

Outliers were detected using the modified Dixon method (Dixon 1983), whereby the largest value (x(n)) is rejected if the distance between it and x(n-1) is more than one third of the range of the sample. That is, reject x(n) if:

$$r = \frac{x(n) - x(n-1)}{x(n) - x(1)} > \frac{1}{3}$$

A non-parametric percentile estimate method, as described by Reed *et al* (1971) and Solberg (1987b) was used to determine the reference limits and reference interval. Briefly, the data was ranked in order of magnitude. To estimate the reference limit below which 95% of the population's serum xanthine oxidase activities would fall, the rank of the upper limit was calculated by:

$$\text{Rank} = 0.95\,(N+1) \qquad \text{(Where N = sample size)}$$

(Solberg and Grasbeck 1989, Hartmann 1983)

An alternative method, described by Merkouriou and Dix (1988) was also used for comparison.

Results

Reference values are displayed in Table 3.1. From the data, the following statistics were calculated:

Mean = 49.0 mU/l

Range = 363.6 mU/l

The reference distribution is displayed in Figure 3.1. A unimodal, positively skewed distribution, with positive kurtosis was present. Logarithmic and square root transformation of the reference distribution did not result in a Gaussian distribution (Figures 3.2 and 3.3).

Rank	Breed	Sex	Age* (Years/Months)	Xanthine Oxidase (mUnits/l
1	NZ Working dog	M	0 / 4	0.0
2	NZ Working dog	M	0 / 4	0.0
3	NZ Working dog	M	0 / 4	0.0
4	NZ Working dog	F	0 / 4	0.0
5	NZ Working dog	F	0 / 4	0.0
6	NZ Working dog	F	0 / 4	0.0
7	NZ Working dog	F	0 / 4	0.0
8	NZ Working dog	F	0 / 4	0.0
9	NZ Working dog	F	0 / 4	0.0
10	Huntaway	F	1 / 2	0.0
11	NZ Working dog	F	0 / 9	0.0
12	Jack Russell terrier	F	1 / 0	0.0
13	NZ Working dog	F	0 / 7	0.0
14	NZ Working dog	F	0 / 7	0.0
15	Poodle	F	3 / 0	0.0
16	Rottweiler cross	M	5 / 0	0.0
17	Fox terrier	F	2 / 6	0.0
18	Huntaway	M	5 / 0	0.0
19	Doberman	M	8 / 6	0.0
20	German Shepherd	F	6 / 0	0.0
21	Border Collie	F	9 / 0	0.0
22	Labrador	F	10/ 0	0.0
23	Fox terrier	M	3 / 6	11.4
24	Doberman	M	6 / 6	22.7
25	Labrador	F	0 / 5	34.1
26	Elkhound	M	8 / 6	34.1
27	Border Collie	M	12/ 0	45.5
28	GSP	F	3 / 6	56.8
29	Poodle	F	3 / 0	79.6
30	German Shepherd	F	7 / 0	79.6
31	Doberman	F	6 / 0	90.9
32	Labrador	F	7 / 6	102.3
33	German Shepherd	F	1 / 0	125.0
34	NZ Working Dog	F	7 / 0	136.4
35	German Shep	M	1 / 0	147.7
36	German Shepherd	M	9 / 0	159.1
37	German Shepherd	M	2 / 0	204.6
38	Golden Retriever	F	13/ 0	215.9
39	Daschund	M	0 / 7	363.6

Table 3.1 Serum Xanthine Oxidase Activity in 39 Clinically Healthy Dogs

NZ = New Zealand, GSP = German Shorthaired Pointer,

M = male (entire or castrated), F = female (entire or speyed)

* Ages greater than one year old are rounded to the nearest 6 months

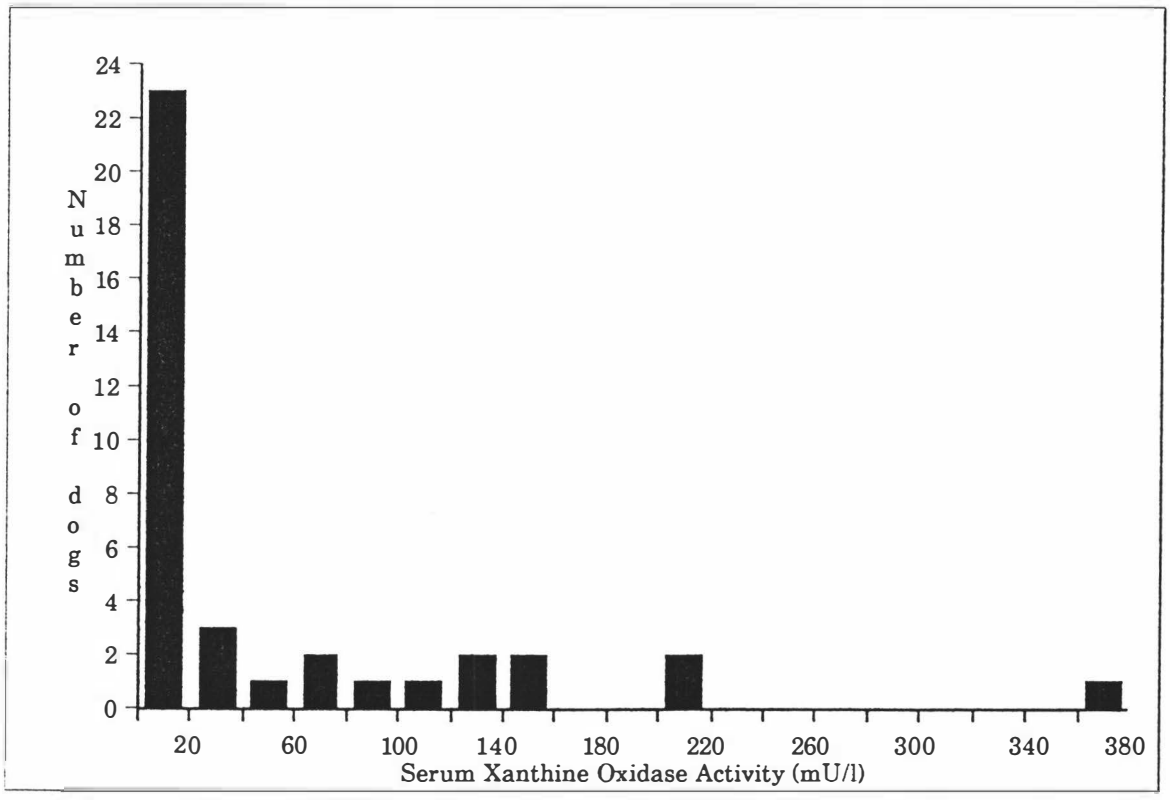

Figure 3.1 Distribution of Serum Xanthine Oxidase Activity in 39 Clinically Healthy Dogs
(Data are presented as the number of dogs falling within each serum xanthine oxidase activity)

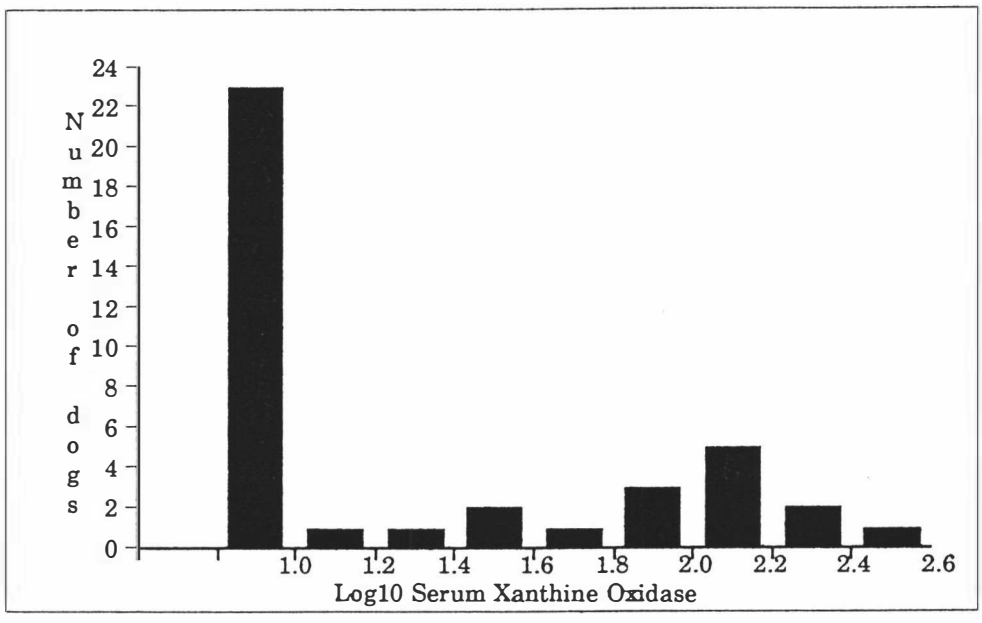

Figure 3.2 Logarithmic Transformation of the Reference Distribution of Serum Xanthine Oxidase Activity in 39 Clinically Healthy Dogs

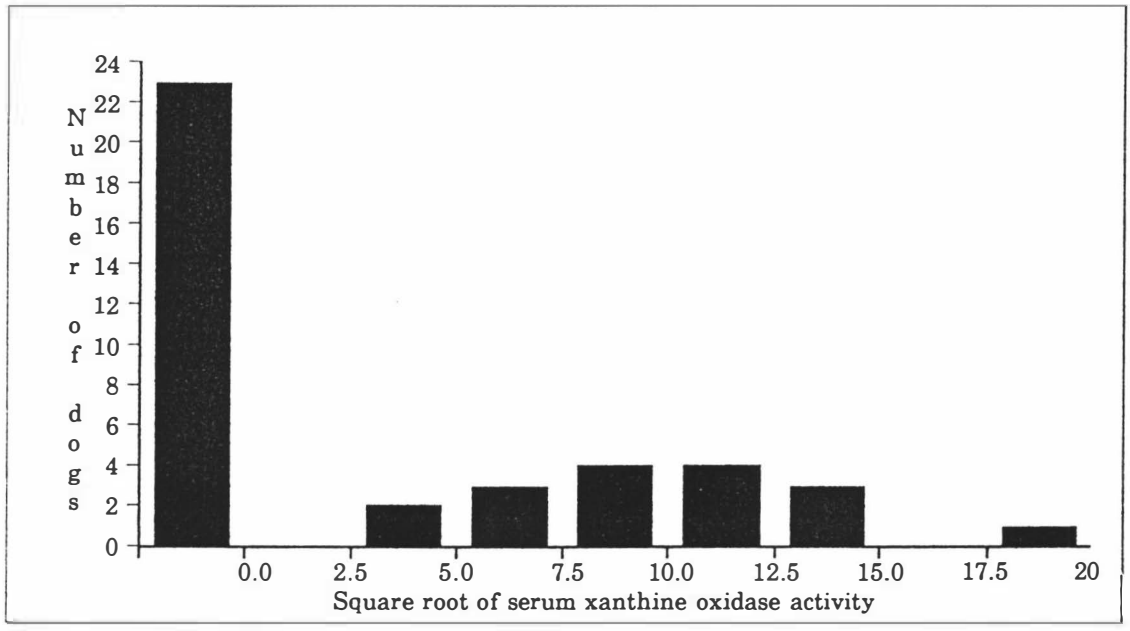

Figure 3.3 Square Root Transformation of the Reference Distribution of Serum Xanthine Oxidase Activity in 39 Clinically Healthy Dogs

The relationship between serum xanthine oxidase activity, age and sex is displayed in Figure 3.4. The mean serum xanthine oxidase activity for the male and female dogs was 70.6 and 36.8 nU/l respectively. There was no significant difference between reference values obtained from male and female dogs (Kruskal Wallis One Way Non-parametric Analysis of Variance Test, Chi-squared = 0.25, p = 0.26). There was no correlation between age and serum xanthine oxidase activity (Pearson Correlation Coefficient = 0.191, p = 0.25).

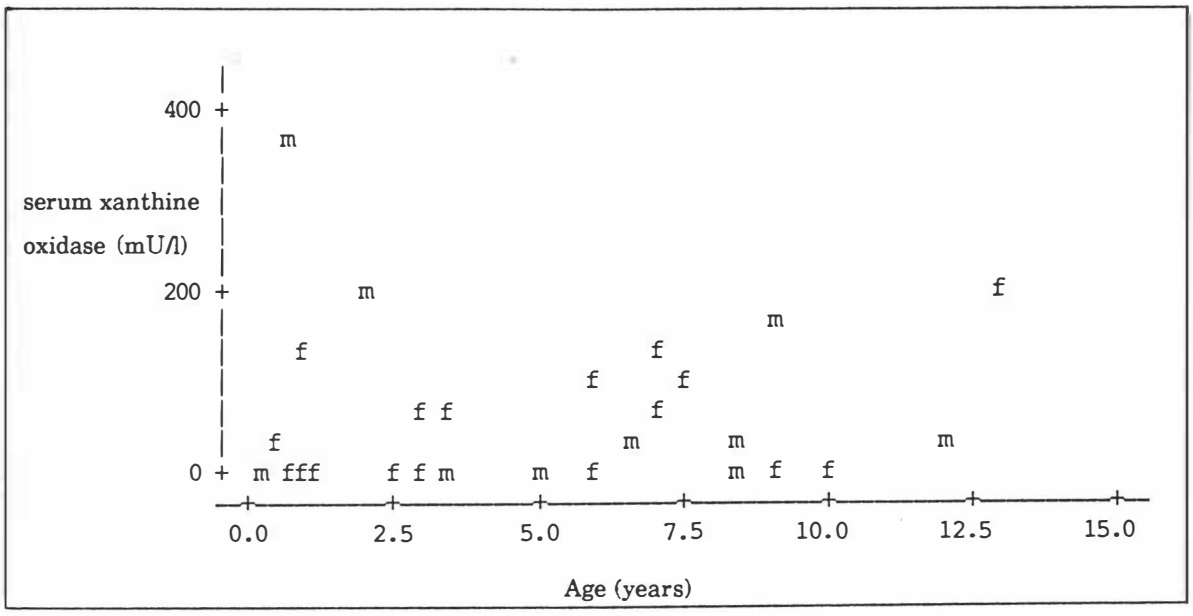

Figure 3.4 Serum Xanthine Oxidase Activity of 39 Clinically Healthy Dogs of Various Ages
M = Male (entire or castrated), F - Female (entire or speyed)

One outlier was identified. The thirty-ninth ranked reference value of 363.6 mU/l, obtained from a 7 month old, male Daschund, was discarded prior to the application of the non-parametric method to determine reference limits (r = 0.41).

Using the percentile estimate method described by Reed *et al* (1971), and Solberg (1987b), the upper fractile (0.95) was calculated to be the thirty-seventh ranked reference value (204.6 mU/l), creating a reference interval of 0 - 204.6 mU/l. A confidence interval for each reference limit could not be calculated due to the relatively small sample size. Using a value versus cumulative percentile plot (Figure 3.5), and the method described by Merkouriou and Dix (1988), the same reference interval was defined, and determined to be approximately 0 - 204.6 mU/l.

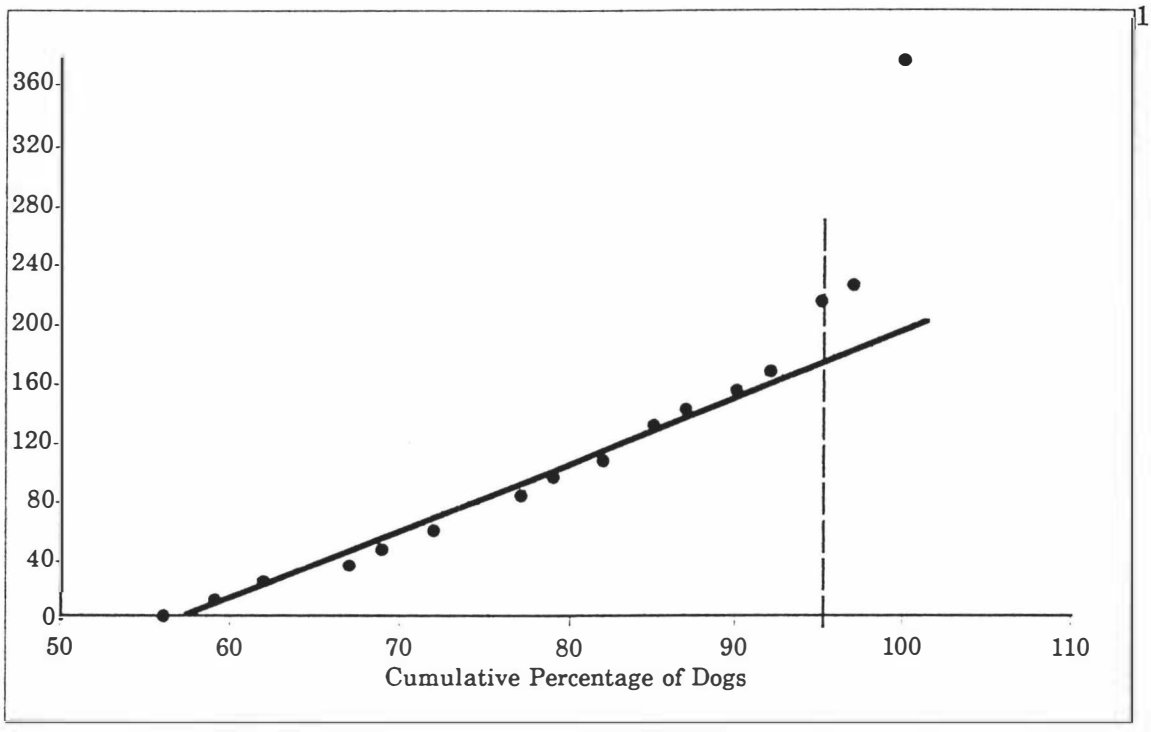

Figure 3.5 Cumulative Percentile Plot of Xanthine Oxidase Activity

The dotted line identifies the percentile and value

where deviation from linearity occurs

Discussion

The mean serum xanthine oxidase activity (49.0 mU/l) was similar to the mean xanthine oxidase activity of three dogs (44 mU/l), reported by Al Khalidi and Chaglassian (1965). Many dogs had no measurable xanthine oxidase activity (56%). Marked variation in the serum xanthine oxidase activity between individual dogs was present, reflected by the large range and standard deviation (Bakes-Martin 1993). The unimodal, positively skewed distribution found in this study is typical of the distribution of other reference values in clinical pathology (Merkouriou and Dix 1988). A positively skewed distribution is asymmetric with an extended right tail (Solberg 1987b).

There are no other studies describing the distribution and range of serum xanthine oxidase activity in dogs to compare with the results of this study. However, measurement of serum xanthine oxidase activity in other species has demonstrated large individual variation. Lochner *et al* (1990), measured the serum xanthine oxidase activity in 34 "healthy" adult horses, using colorimetry. Serum xanthine oxidase activity ranged from 0 to 126 mU/l, with a mean of 44.95, and standard deviation of 21.05 mU/l. Their data approximated a Gaussian distribution. Giler *et al* (1975) used a radioisotopic method to measure the serum xanthine oxidase activity in 20 "healthy" people. The mean serum xanthine oxidase activity was 1.35 mU/l, the standard deviation was 2.15 mU/l and the range was 6.58 mU/l. The distribution was not described. In another study using a similar type of assay, the serum xanthine oxidase activity in 25 human "volunteers", had a mean of 6.7 mU/l, and a range of 34.6 mU/l. A similar reference distribution to that obtained in the present study, was seen (McCarthy and Long 1976). Ramboer *et al* (1972) measured the serum xanthine oxidase activity in 36 "healthy" people using a spectrophotometric assay of uric acid production. Activities ranging from 0 - 9 mU/l were measured and a large percentage of individuals (53%) had no measurable xanthine oxidase activity in the serum. Majkic-Singh *et al* (1987) used a different spectrophotometric method (using 2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonate), to measure serum xanthine oxidase activity in 83 "healthy" people and found a mean of 252 mU/l, a standard deviation of 242 mU/l, and estimated the reference interval to be 0-1200 mU/l using the same non-parametric method as used in the present study. Again, many people had no measurable xanthine oxidase activity (38%) and the distribution was not Gaussian. The marked variation in human serum xanthine oxidase activity measured by different authors probably reflects the different types and conditions of assay (such as temperature and pH), and emphasises why it is necessary to determine a reference interval for each type of assay, under standardised conditions.

Many biological factors may influence the activity of an enzyme in serum, and contribute to biological variation (Statland and Winkel 1981)(Table 3.2).

Some Potential Factors Influencing Serum Enzyme Activities*
Age
Altitude
Body mass
Breed
Chronobiological rhythms
Diet
Disease
Drugs
Environment (humidity/temperature)
Exercise
Exposure to toxic agents, radiation
Genetic factors
Physical fitness
Pregnancy
Puberty
Sex - male, female, entire or desexed
Site of specimen collection
Stress at collection

Table 3.2 Potential Factors Influencing Serum Enzyme Activities

* Derived from Solberg and Grasbeck 1989

From this study it appears that age and sex do not influence the serum xanthine oxidase activity in dogs. Similarly, serum xanthine oxidase activity is not correlated with age or sex in people (McCarthy and Long 1976). Insufficient numbers of each breed of dog were present to detect an influence of breed on serum xanthine oxidase activity.

Exercise may influence the serum xanthine oxidase activity in dogs. Plasma xanthine oxidase activity increased rapidly after the onset of exercise in horses, accompanied by an increase in

plasma uric acid concentration. The intensity of exercise had only a minor influence on the elevation in xanthine oxidase activity (Rasanen *et al* 1996). Xanthine oxidase is found in skeletal muscle (Hellsten-Westing 1993, Rasanen *et al* 1993), particularly within capillary endothelial cells (Jarasch *et al* 1986). Increased oxidative stress during intense exercise may cause ischaemia of muscle, resulting in the conversion of xanthine dehydrogenase to xanthine oxidase (Stirpe and Della Corte 1969). The source of the increased circulating xanthine oxidase was proposed to be the surface or cytosol of capillary endothelial cells, but the precise mechanism of release is unknown (Rasanen *et al* 1996). Exercise can cause elevations in other serum enzyme activities. For example, moderate exercise in people can increase the serum activity of creatine phosphokinase and alanine aminotransferase (Leppanen and Grasbeck 1988). Therefore, the effect of exercise may explain the relatively high xanthine oxidase activities found in some apparently "healthy" dogs in this study. Moreover, some dogs become extremely excited and agitated in the environment of the veterinary clinic, and the increased muscle activity associated with this physiologic state may have resulted in an elevated serum xanthine oxidase activity in an analogous manner to exercise. It was not determined whether the dogs in the present study were exercised prior to the collection of blood.

The effect of diet on xanthine oxidase activity, in particular the consumption of bovine milk, was investigated in pigs and people by McCarthy and Long (1976). Prior to this study it was thought that consumption of milk, which is rich in xanthine oxidase (Batelli *et al* 1973), may lead to absorption of large amounts of xanthine oxidase from the gut, deposition in the walls of blood vessels, and increased serum xanthine oxidase activity. However, the serum xanthine oxidase activity was not correlated to the average daily milk consumption in people, and serum xanthine oxidase activity was undetectable in pigs fed large volumes of milk.

Subclinical disease may lead to the inclusion of individuals in the reference sample population, that are not true representatives of the desired reference population (in this study, all healthy dogs in New Zealand). The concept of "health" is difficult to define, and unlike many other descriptive terms, it is relative, rather than absolute (PetitClerc and Solberg 1987). In this study, the definition of health provided by Solberg and Grasbeck (1989) was considered - "health is characterised by a minimum of subjective feelings and objective signs of disease,

ssessed in relation to the social situation of the subject and the purpose of the medical ctivity". Dogs evaluated in this study were subjectively "healthy", based on a history and hysical examination, but laboratory tests were not performed to screen each dog. It is possible hat some reference values were obtained from "unhealthy" dogs with undetectable conditions for example, mild gastrointestinal or hepatic disease), falsely elevating the measured serum anthine oxidase activity. Elevations in serum xanthine oxidase activity have been described in uman patients with viral hepatitis and hepatic neoplasia (Ramboer *et al* 1972, Shamma'a *et al* 973). Many of the potential factors listed in Table 3.2 remain uninvestigated in relation to erum xanthine oxidase activity, and these may be worthy of investigation in the future.

he method of selection of reference individuals will also influence the values obtained. Selection hould be random, in that each individual in the population has the same chance of selection PetitClerc and Solberg 1987). The method of selection used in this study was biased towards logs belonging to clients and staff of the Massey University Veterinary Clinic (MUVC), dogs who vere vaccinated, and dogs bred by the Animal Health Services Centre, many of which were genetically related. However, given that the reference values were obtained for the purpose of comparison with sick dogs presented to the MUVC, this bias is unlikely to be important.

Procedures for the collection, storage and analysis of the serum were standardised, to minimise the effect of *in vitro* factors, such as the effect of freezing, thawing and the timing of centrifugation, on the activity of the enzyme in serum (Alstrom *et al* 1975).

The first step in the assessment of reference values is to view their distribution, displayed on a histogram (Solberg and Grasbeck 1989). An outlier may be suspected at this stage. Outliers are values which deviate so much from the other values as to arouse suspicion (Hawkins 1980). They can arise due to contamination of the data with "false" values (for example, a subclinically diseased dog), or occur as a result of an error in analysis, or be "real" values, and merely reflect the true distribution. Several statistical methods have been described to identify possible outliers (Healy 1979, Hawkins 1980, Barnett 1994), but many are only applicable if the reference distribution is Gaussian. The IFCC recommends the use of the modified Dixon Test to identify outliers (Dixon 1953, Solberg 1987b). This identifies a value as an outlier if the

difference between the largest (or smallest) value in the distribution and the next largest (or smallest) value is greater than one third of the difference between the maximum and minimum values of the distribution.

Once an outlier is detected, a possible explanation should be sought, before a decision is made whether to include, correct or discard the value. The handling of outliers seems to be influenced more by the opinion of the analyst, rather than mathematics or science, and the topic is debatable (Strike 1991, Solberg and Grasbeck 1989). Discarding outliers carries a risk of removing "good" data (Hawkins 1980). The outlier identified in this study (363.6 mU/l) was measured in a 7 month old, male Daschund. As mentioned above, this could be a true value, or one elevated by exercise, experimental error or subclinical disease. Insufficient serum was collected to repeat the assay, and the dog remained subjectively healthy for greater than 12 months.

Reference limits and their confidence intervals may be determined using parametric or nonparametric statistical methods. Parametric methods are commonly used, and assume that the distribution is Gaussian (Solberg 1987c). They cannot be used if the distribution is not Gaussian, but are often misused in this manner (Solberg 1987b). If a non-Gaussian distribution can be converted to the Gaussian form using logarithmic or square root transformations, then parametric methods can be used (Strike 1991, Solberg and Grasbeck 1989). The reference distribution of serum xanthine oxidase in healthy dogs in this study could not be transformed to a Gaussian distribution using these methods, so a nonparametric method was chosen. Nonparametric methods can be used with all types of distribution (Solberg 1987c). Parametric and nonparametric methods have been compared using experimentally derived and hypothetical data, and although there is debate as to which method is the best (Reed *et al* 1971, Schultz *et al* 1985, Boyd and Lacher 1982, Linnet 1987) it is generally believed that both types of methods perform well when used appropriately (Solberg and Grasbeck 1989).

It is conventional that the reference interval contains the central 95% of the reference population values (Harris 1981, Bush 1991), but an asymmetric location of the reference interval may be more appropriate in some situations (Solberg 1987b), including the situation in this

study, where only an elevation in serum xanthine oxidase activity is of interest. When reference limits are stated it is preferable that they are accompanied by a confidence interval around each reference limit (Solberg 1987b). Estimation of reference limits becomes less precise as the sample size decreases, reflected by wide confidence intervals. It is suggested that at least 100 reference values be measured for setting reference intervals (Bakes-Martin 1993). Due to the sample size in this study, a precise 90% confidence interval could not be calculated but, given the sample size it can be estimated that the reference interval will exclude a minimum of 0.1% and a maximum of 7.5% of the population (Reed *et al* 1971).

Merkouriou and Dix's method (1988) appears useful for skewed unimodal distributions, and is based on the principle that typical values (that is, those within a suitable reference interval) exhibit a linear relationship with cumulative percentiles. Deviation from linearity identifies values that are atypical. This method has not yet been critically reviewed and it should be used with care until such a time.

Finally, it should be remembered that not all values from "healthy" individuals will fall within the reference interval, and that some values from "unhealthy" animals will fall within the interval (Bush 1991), highlighting that the interpretation of a laboratory test should be performed in light of the history, physical examination findings, and other diagnostic tests.

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Chapter 4

Serum Xanthine Oxidase Activity in Dogs Undergoing Ischaemia-Reperfusion Injury

Introduction

Ischaemia-reperfusion injury can lead to the development of serious complications such as the systemic inflammatory response syndrome (SIRS) (Carden *et al* 1993), acute respiratory distress syndrome (ARDS) (Welbourn *et al* 1991) , disseminated intravascular coagulation (DIC) (Yoshikawa 1990), and multiple organ dysfunction syndrome (MODS) (Badylak *et al* 1986). These complications are usually associated with a poor prognosis (Haskins 1994).

The pulmonary pathology in ARDS is centred around microvascular injury, and is similar to that described at the actual site of ischaemia and reperfusion (Lefer and Lefer 1993). Both locally, and in the lungs, microvascular injury leading to increased capillary permeability, infiltration of inflammatory cells and parenchymal cell injury are seen (Bishop *et al* 1987, Zimmerman *et al* 1990, Koike *et al* 1993). There is much experimental evidence to implicate oxygen derived free radicals (ODFRs), derived from the xanthine oxidase system, in the pathogenesis of the local tissue injury. For example, the experimental use of xanthine oxidase inhibitors, allopurinol and oxypurinol (Akizuki *et al* 1985, Grisham *et al* 1986, Adkison *et al* 1986, Svensson *et al* 1987), ODFR scavengers, such as catalase and superoxide dismutase (Parks *et al* 1982, Parks and Granger 1983, Badylak *et al* 1987), or tungsten supplemented Molybdenum-free diets, to inactivate xanthine oxidase (Deitch *et al* 1988, Nielsen *et al* 1996), has attenuated or prevented the local tissue injury associated with ischaemia and reperfusion of a variety of organ systems. Also, the addition of xanthine oxidase and substrate to the perfusate of an *in vitro* preparation of non-ischaemic intestine, caused similar pathology to that seen in ischaemia- reperfusion injury (Grogaard *et al* 1982, Parks *et al* 1984).

There is also evidence that ODFRs and xanthine oxidase are involved in the pathogenesis of ARDS and DIC. Decamp and Demling (1988) demonstrated that lipid peroxidation occurs in

ARDS, and polymorphonuclear cell infiltration into the lung can be reduced by xanthine oxidase inhibition (Adkins and Taylor 1990). Nielsen *et al* (1996), showed that pulmonary oedema developed in rabbits following hepatoenteric ischaemia-reperfusion, and that this was decreased in rabbits by xanthine oxidase inactivation.

Disseminated intravascular coagulation can be triggered following exposure of subendothelial collagen, secondary to endothelial damage (Weinbroum *et al* 1995). The cytotoxic effects of xanthine oxidase on cultured endothelial cells have been demonstrated by Yokohama *et al* (1990), and Barnard and Matalon (1992), and in 1990, Yoshikawa showed that ODFR scavengers prevented the development of DIC in rats with experimental endotoxic shock. Oxygen derived free radicals can also cause platelet aggregation and activation by stimulating the production of Platelet Activating Factor (Kubes and Granger 1992, Kuwano *et al* 1996), further contributing to the development of DIC.

The occurrence of widespread vascular endothelial injury, and tissue injury at a site distant from the actual site of ischaemia and reperfusion, suggests that a circulating mediator is involved in the pathophysiology of ARDS and DIC following such injury. There is evidence supporting xanthine oxidase as a possible mediator. Weinbroum *et al* (1995), induced pulmonary oedema in isolated non-ischaemic rat lungs, by perfusing them with "waste" perfusate, obtained from isolated ischaemic-reperfused rat liver. Xanthine oxidase activity was high in the perfusate, and the addition of allopurinol to the perfusate attenuated the increase in pulmonary capillary permeability and resultant oedema. In another study, intravenous administration of xanthine oxidase in rats induced white blood cell infiltration into the lung, however, pulmonary oedema was not seen (Terada *et al* 1992), therefore xanthine oxidase may not be the sole mediator of ARDS. Other substances such as cytokines and complement have also been suggested as possible mediators (Welbourn *et al* 1991, Weinbroum *et al* 1995).

Circulating xanthine oxidase activity has been shown to increase following *in vivo* ischaemia-reperfusion injury of a variety of organs including the liver (Friedl *et al* 1990, Nielsen *et al* 1996), intestine (Caty *et al* 1990, Terada *et al* 1992), and distal limb of laboratory animals (Punch *et al* 1992). Elevation of circulating xanthine oxidase activity has also been shown to occur in *in vivo*

models of endotoxic and haemorrhagic shock in horses and rats respectively (Lochner *et al* 1990, Tan *et al* 1993). These conditions potentially lead to ischaemia of the liver and intestine, which are rich in xanthine dehydrogenase/oxidase (Al Khalidi and Chaglassian 1965). Ischaemia leads to the conversion of the relatively "benign" xanthine dehydrogenase form, to the ODFR producing oxidase form (Engerson *et al* 1989), which could be released into the systemic circulation upon reperfusion. Large amounts of xanthine oxidase are released from the liver following *in vitro* hepatic ischaemia and reperfusion, accompanied by a large decrease in liver xanthine oxidase activity (Yokoyama and Parks 1988, Yokoyama *et al* 1990, Weinbroum *et al* 1995).

There have been few reports regarding the circulating xanthine oxidase activity of patients with ischaemic disorders, ARDS or DIC. Circulating xanthine oxidase activity is elevated in humans with ARDS (Weiland *et al* 1986, Grum *et al* 1991), or sepsis syndrome (similar to SIRS) (Galley *et al* 1996). An increase in plasma xanthine oxidase activity has also been observed following the release of a tourniquet applied to human limbs (Friedl *et al* 1991) and following release of an aortic cross-clamp placed to facilitate aortic surgery in one person (Tan *et al* 1995).

Serum xanthine oxidase activity has not been measured in sick dogs with diseases which may involve ischaemia-reperfusion injury. In the present study it was hypothesised that:
(1) the serum xanthine oxidase oxidase activity in dogs with these diseases, would be greater than that of "healthy" dogs,
(2) the xanthine oxidase activity in serum collected following reperfusion would be higher than that in serum collected prior to reperfusion
(3) the serum xanthine oxidase activity would be greater in dogs with clinical signs of severe cardiovascular compromise, than in dogs with milder clinical signs, and
(4) the serum xanthine oxidase activity in dogs that died would be greater than dogs that survived.
The purpose of this study was to test these hypotheses by measuring serum xanthine oxidase activity prior to, and following reperfusion, in dogs with diseases resulting in poor tissue perfusion.

Materials and Methods

Animals

Thirty dogs presented to the Massey University Veterinary Clinic, or the Taita Veterinary Hospital, with the following conditions were used in this study: gastric dilatation (n=3), gastric dilatation-volvulus (GDV) (n=7), intestinal foreign body (n=3), parvoviral enteritis (n=5), idiopathic haemorrhagic gastroenteritis (n=1), pancreatitis (n=2), pyometra (n=2), hypovolaemic haemorrhagic shock, secondary to anticoagulant toxicity (n=2), systemic inflammatory response syndrome (SIRS) secondary to sepsis (n=4), and heatstroke (n=1). A variety of breeds and age were represented (see table 4.1)

The heart rate, pulse quality, mucous membrane colour, and capillary refill time at initial examination in the veterinary clinic were recorded and used to grade the severity of cardiovascular compromise, using the following guidelines:

(a) mild - heart rate < 100 beats per minute for giant breeds (eg Great Dane)
< 140 beats per minute for medium sized breeds (eg Labrador)
< 180 beats per minute for puppies and toy breeds (eg Papillion)
good pulse quality
pink mucous membranes
capillary refill time 1-2 seconds

(b) moderate - heart rate greater than above
good pulse quality
pale mucous membranes
capillary refill time 2-3 seconds

(c) severe - heart rate greater than above
weak or bounding pulse
"injected" or "muddy" mucous membranes
capillary refill time <1 second or > 3 seconds

The dogs were hospitalised and treated appropriately for the disease condition affecting them.

Treatment always included intravenous fluids (crystalloids, and/or colloids and/or blood products), and sometimes included surgery (for example, foreign body removal, gastric decompression and gastropexy for GDV, ovariohysterectomy for pyometra), and medications (for example, antibiotics, corticosteroids, H_2 blockers, heparin). The final outcome (survival or death) was recorded. All procedures were approved by the Massey University Animal Ethics Committee.

Serum

One to five millilitres of blood was collected from a peripheral vein (cephalic, jugular or saphenous veins) prior to treatment (the pre-reperfusion sample), and one hour following reperfusion (the post-reperfusion sample). In some dogs, only one serum sample was able to be collected, as treatment had already been initiated. The time of reperfusion was defined as the time at which the administration of intravenous fluids, equivalent to one blood volume, was complete (60-90 ml/kg body weight of crystalloid, 10-20 ml/kg of colloid or blood products (Tobias and Schertel 1992), or the time of decompression and replacement of the stomach to the correct position in dogs with GDV. Serum was harvested and stored as described in Chapter 3.

Materials and Solutions

The source of chemicals, and preparation of solutions was as described in Chapter 1.

Xanthine Oxidase Assay

Each serum sample was assayed in duplicate for xanthine oxidase activity, using the spectrophotometric method described in Chapter 1. Two hundred to 400 µl of serum was used in each assay. The serum xanthine oxidase activity was expressed as milliunits per litre (mU/l).

Statistics

The mean serum xanthine oxidase activity prior to and following reperfusion was calculated. The Kruskal Wallis one way nonparametric analysis of variance (KW-ANOVA) test was used to compare pre- and post-reperfusion serum xanthine oxidase activities with reference values obtained in Chapter 3. The sign test was used to compare pre-reperfusion and post-reperfusion

serum xanthine oxidase activities. The severity of clinical signs and outcome of the disease (survival or death), were compared with pre-reperfusion and post-reperfusion xanthine oxidase activities, and the change in xanthine oxidase activity associated with reperfusion, using the KW-ANOVA test.

Results

The pre-reperfusion and post-reperfusion serum xanthine oxidase activities, the diagnosis made, the severity of clinical signs of cardiovascular compromise, and outcome (survival or death) are shown in Table 4.1.

The mean serum xanthine oxidase activities pre- and post-reperfusion were 95.0 and 151.4 mU/l respectively. Both pre- and post-reperfusion serum xanthine oxidase activities were significantly higher than the serum xanthine oxidase activities of the reference individuals (KW - ANOVA Chi-Squared = 6.66, $p = 0.01$, and Chi-Squared = 16.59 $p = 0.0001$, respectively). The serum xanthine oxidase activities following reperfusion were significantly higher than prior to reperfusion (Sign test $p = 0.008$, $n = 20$).

The clinical signs of cardiovascular compromise were mild, moderate or severe in 20%, 37% and 43% of dogs, respectively. The mean serum xanthine oxidase activities in dogs with mild, moderate or severe signs of cardiovascular compromise prior to reperfusion were 22.7, 127.5 and 178.3 mU/l, respectively, and following reperfusion were 90.9, 56.8 and 130.2 mU/l, respectively. The serum xanthine oxidase activity was not significantly different between groups, both prior to, and following reperfusion (KW-ANOVA Chi-Squared = 3.87, $p = 0.14$, and Chi-Squared = 2.16, $p = 0.34$, respectively).

Forty-one percent of the dogs died as a result of the ischaemic disorder or secondary complications. The mean serum xanthine oxidase activity for dogs that survived was 100.0 mU/l pre-reperfusion, and 110.5 mU/l post-reperfusion. For dogs that died, the mean serum

xanthine oxidase activity was 95.2 mU/l pre-reperfusion, and 99.5 mU/l post-reperfusion. There was no significant difference between the serum xanthine oxidase activities measured (both pre- and post-reperfusion) in dogs that survived, compared with those that died (KW-ANOVA Chi-Squared = 0.58, p = 0.45, KW-ANOVA Chi-Squared = 0.15, p = 0.7, respectively) nor was there a significant difference between the change in xanthine oxidase activity with reperfusion, in dogs that survived compared with those that died (KW-ANOVA Chi-Squared = 0.11, p = 0.74).

Case number	Diagnosis	Breed	Sex	Age (Yrs/mths)	XO mU/l Pre	XO mU/l Post	Severity	Outcome
1	Gastric dilatation	Labrador	F	10/0	34.1	102.3	Mild	+
2	Gastric dilatation	Border Collie	M	9/0	34.1	159.1	Moderate	-
3	Gastric dilatation	Huntaway	M	2/5	22.7	68.2	Severe	-
4	GDV	Huntaway	M	3/0	0.0	0.0	Moderate	+
5	GDV	Borzoi	M	10/0	45.5	181.8	Severe	-
6	GDV	Boxer	F	12/0	68.2	363.6	Severe	-
7	GDV	Irish Setter	F	9/0	159.1	159.1	Severe	-
8	GDV	Rottweiler Cross	M	4/0	N/A	340.9	Severe	-
9	GDV	Standard Poodle	F	8/4	90.9	340.9	Severe	-
10	Chronic GDV	Great Dane	F	5/0	22.7	N/A	Mild	+
11	Intestinal FB	NZ Working Dog	M	2/0	90.9	113.6	Severe	+
12	Intestinal FB	Staffordshire Terrier	M	4/0	0.0	45.5	Mild	+
13	Intestinal FB	German Shepherd	M	3/0	125.0	159.1	Severe	+
14	Parvovirus	Rottweiler	F	0/3	N/A	159.1	Mild	+
15	Parvovirus	Rottweiler	F	0/4	45.5	250.0	Moderate	+
16	Parvovirus	Pitbull Cross	F	0/7	204.5	227.3	Moderate	+
17	Parvovirus	Staffordshire Terrier	M	0/8	N/A	22.7	Moderate	+
18	Parvovirus	Rottweiler Cross	M	1/0	N/A	113.6	Moderate	+
19	HGE	Papillion	M	4/0	90.9	0.0	Severe	+
20	Pancreatitis	Labrador Cross	F	11/0	0.0	22.7	Mild	+
21	Pancreatitis	Daschund	F	6/0	250.0	N/A	Mild	Euthanased
22	Pyometra	English Bull Terrier	F	8/0	68.2	272.7	Moderate	+
23	Pyometra	Rottweiler	F	10/0	386.4	272.7	Severe	+
24	Hypovolaemic shock	Bull Mastiff	M	1/3	N/A	0.0	Moderate	+
25	Hypovolaemic shock	NZ Working Dog	M	14/0	N/A	136.4	Severe	-
26	SIRS	Corgi	M	12/0	N/A	204.5	Moderate	-
27	SIRS	Labrador	F	9/0	0.0	113.6	Moderate	+
28	SIRS	NZ Working Dog	M	0/9	N/A	113.6	Moderate	-
29	SIRS	Labrador	F	4/0	136.4	159.1	Severe	-
30	Heatstroke	Pitbull Terrier	M	4/0	215.9	22.7	Severe	-

Table 4.1 Pre-reperfusion and Post-reperfusion Serum Xanthine Oxidase (XO) Activity, Severity of Clinical Signs of Cardiovascular Compromise and Outcome in 30 Sick Dogs.

GDV = gastric dilatation-volvulus, FB = foreign body, HGE = haemorrhagic gastroenteritis, SIRS = systemic inflammatory response syndrome, NZ = New Zealand, M = male (entire or castrated),F = female (entire or speyed), Pre = pre-reperfusion blood sample, Post = post-reperfusion sample, N/A = not assayed, + = survived, - = died.

Discussion

Most of the dogs with ischaemic disorders had higher serum xanthine oxidase activities than 'healthy" dogs. The mean serum xanthine oxidase activity following reperfusion was three times greater than the mean activity of the "healthy" dogs, described in Chapter 3. Increases in the circulating xanthine oxidase activity of a similar magnitude, have been described following experimental intestinal, hepatic, and distal limb ischaemia-reperfusion injury in rats (Terada *et al* 1992, Friedl *et al* 1990, Punch *et al* 1992).

Some dogs had a very low, or undetectable serum xanthine oxidase activity, in spite of their ischaemic disorder. The amount of xanthine oxidase released into the circulation during ischaemia-reperfusion could have been affected by a variety of factors, such as the duration and severity of ischaemia, and the effects of anaesthesia, surgery or drugs. The severity of the local tissue injury is affected by the duration and severity of the ischaemia (Chiu *et al* 1970). The liver and intestine appear to have an ability to extract greater percentages of the oxygen delivered to the tissue when the blood supply is decreased, compared with extraction in "normal" conditions. This provides a degree of protection against ischaemia (Adkison *et al* 1986, Bulkley *et al* 1985). More prolonged periods of ischaemia would be expected to cause more parenchymal cell damage and greater increases in capillary permeability, resulting in a larger amount of xanthine oxidase reaching the systemic circulation. Furthermore, short periods of ischaemia may be insufficient for significant conversion of xanthine dehydrogenase to oxidase. Fifty percent conversion takes at least 2 hours in the rat intestine and liver (Engerson *et al* 1987, Parks *et al* 1988). The possible effect of duration of ischaemia on serum xanthine oxidase activity may be illustrated by a comparison of the serum xanthine oxidase activities of cases 4 and 9. Case 4 (GDV), was presented to the veterinary clinic within 30 minutes of developing clinical signs of abdominal distension and retching. Serum xanthine oxidase was undetectable. In contrast, case 9, also suffering from GDV, was presented after approximately 12 hours of showing clinical signs, and had a very high serum xanthine oxidase activity (340.9 mU/l).

The effect of anaesthesia, surgery or drugs on the serum xanthine oxidase activity in dogs is

unknown. Elevation of serum xanthine oxidase activity has been reported in rats and humans following halothane inhalation anaesthesia, possibly secondary to the hepatotoxic effects of this drug (Giler *et al* 1976b, Giler *et al* 1977), or perhaps periods of hypoxia during anaesthesia. Surgical manipulation of the liver and upper gastrointestinal tract has resulted in elevation in serum xanthine oxidase activity in humans (Giler *et al* 1976a). In the present study, 12 dogs underwent a surgical procedure (under anaesthesia) as part of their treatment. The mean serum xanthine oxidase activity appeared similar in these dogs, to that of dogs not undergoing surgery (surgical cases 189.4 mU/l, non-surgical 163.0 mU/l).

A variety of drugs were used in conjunction with intravenous fluid therapy and surgery in this study. Heparin was used for the treatment of DIC in 7 cases. Heparin is known to compete with xanthine oxidase for glycosaminoglycans binding sites on endothelial cells (Hiebert and Liu 1991), and could theoretically dislodge xanthine oxidase, increasing the circulating activity of this enzyme. However, Yamamoto *et al* (1996) found that serum xanthine oxidase activity did not rise following intravenous administration of a single dose of heparin (30U/kg), in humans . In the present study there appeared to be a trend towards a higher serum xanthine oxidase activity in dogs treated with heparin (mean 196.4 mU/l), than in dogs that did not receive heparin (mean 118.1 mU/l). Larger doses of heparin (100-150 Units/kg every 6-8 hours) were used in the present study than in the human study, increasing the likelihood of competition with xanthine oxidase for the endothelial binding sites. However, it is noteworthy that many dogs with elevated serum xanthine oxidase activity did not receive heparin. Furthermore, heparin was exclusively used in dogs with DIC, and it is possible that pre-existing elevated xanthine oxidase activity may have contributed to the vascular damage and DIC, that necessitated the use of heparin.

The pre-reperfusion serum xanthine oxidase activities were significantly higher than those of "healthy" dogs. However, Caty *et al* (1990) found that the circulating xanthine oxidase activity in rats with complete intestinal ischaemia did not increase during the ischaemic phase. A likely explanation for the increase in pretreatment activities, is that dogs in the present study were primarily undergoing low-flow, rather than no-flow ischaemia. This allows some oxygen entry into the tissue and ODFR production by xanthine oxidase, followed by the deleterious effects of

radicals on tissue, and xanthine oxidase release into the circulation. Xanthine oxidase activity was usually higher in post-reperfusion serum specimens, compared with specimens obtained prior to treatment. This was expected to occur as it has been shown that a large amount of xanthine oxidase is released from ischaemic rat liver, after merely 1 to 5 minutes of reperfusion (Weinbroum *et al* 1995, Friedl *et al* 1990). In previous studies, xanthine dehydrogenase has also been observed to be released into the circulation, and this has been shown to be converted to the oxidase form by sulphydryl oxidation, within seconds of release into plasma (Arnold *et al* 1994). The time of peak circulating xanthine oxidase activity in rats with experimental hepatic and intestinal ischaemia-reperfusion injury, has ranged from 10 minutes to 60 minutes following reperfusion, respectively (Friedl *et al* 1990, Caty *et al* 1990). The change in serum xanthine oxidase activity over time in dogs with ischaemic disorders has not been evaluated, and serum samples may not have been collected when xanthine oxidase activity was maximum.

The serum xanthine oxidase activity decreased following reperfusion in three dogs (cases 19, 23 and 30). In case 23, the post-reperfusion enzyme activity was still very high. Case 30 was referred to the Massey University Veterinary Clinic (MUVC) for treatment of heatstroke, and received intravenous fluids prior to transportation, hence the "pre-reperfusion" serum sample was not truly taken prior to reperfusion. The decline after further fluid therapy may represent a decrease in the amount of xanthine oxidase released from ischaemic organs over time, or be secondary to haemodilution due to further intravenous fluid administration. Case 19 (diagnosed with HGE), was presented to the MUVC within 45 minutes of developing clinical signs of depression, haemorrhagic diarrhoea and vomiting, and was severely haemoconcentrated. The relatively short period of ischaemia may have limited the amount of xanthine dehydrogenase to oxidase conversion, and ischaemic tissue damage, and the dilution effect of fluid therapy may have also contributed to the undetectable xanthine oxidase activity in the post-reperfusion sample.

The highest serum xanthine oxidase activities were found in three dogs with GDV (cases 6, 8 and 9), and one dog with a pyometra (case 23). However, it is not possible to make generalisations regarding the diseases in which serum xanthine oxidase activities are high, or low using this data, due to the small numbers of dogs suffering from each condition.

There was a statistically insignificant trend for serum xanthine oxidase activity to be higher in dogs with more severe clinical signs relating to the cardiovascular system. The lack of statistical significance may be due to lack of statistical power, and therefore further studies with larger numbers of subjects may be of value.

Five dogs in the present study developed respiratory failure, associated with ARDS (cases 2, 5, 26, 29 and 30). The mean serum xanthine oxidase activity for the dogs which developed ARDS was 184.1 mU/l, compared with a mean activity of 117.2 mU/l for dogs that survived the ischaemic disorder. Nielsen *et al* (1996) showed that in rabbits, the likelihood of the development of pulmonary oedema secondary to experimental hepatoenteric ischaemia-reperfusion was correlated to the plasma xanthine oxidase activity. Further evaluation in dogs would be useful.

A large proportion of dogs died despite therapy, highlighting the severity of these ischaemic conditions. One dog (case 21) was euthanased due to other reasons, and was expected to survive, so was not included in the mortality calculations. The serum xanthine oxidase activity does not appear to be useful as a prognostic indicator for death or survival, but further studies using larger numbers of dogs may be useful to further investigate any relationship between serum xanthine oxidase and outcome. Interestingly, the dog with acute GDV with no detectable xanthine oxidase activity (case 4), was the only dog with GDV that survived in this study.

This study found that dogs with ischaemic disorders had high activities of serum xanthine oxidase, compared with apparently "healthy" dogs. This finding suggests that circulating xanthine oxidase may play a role in the pathophysiology of ARDS, DIC and other complications of ischaemia-reperfusion injury, and that allopurinol therapy may be useful in preventing, or reducing the severity, of these complications. Circulating xanthine oxidase could react with purine substrates in the plasma, which are found in high concentrations during reperfusion (Frank *et al* 1988), producing ODFRs at many sites throughout the body (Tan *et al* 1993). These ODFRs could directly damage the vascular endothelium, or via their chemotactic effects, attract inflammatory cells, which may damage the endothelium (Weinbroum *et al* 1995). Xanthine oxidase is able to bind to sulphated glycosaminoglycans in cell membranes (White *et al*

1996), particularly of vascular endothelial cells (Freeman *et al* 1991), but also in pulmonary interstitial and alveolar epithelial cells (Karlsson *et al* 1988). Weinbroum *et al* (1995), found that perfusion of isolated rat lung with a solution containing high xanthine oxidase resulted in an increase in xanthine oxidase activity in the pulmonary tissue. It appears that the lungs can selectively take up xanthine oxidase, probably as a result of glycosaminoglycan binding, and this would result in a much higher local xanthine oxidase activity than that found in the circulation (White *et al* 1996). This may explain why the lungs are a common site of secondary injury.

The ability of allopurinol to prevent, or attenuate, the complications of ischaemia-reperfusion injury in distant organs, has received little attention. Most studies have focused upon the ability of allopurinol to reduce the acute tissue injury at the site of ischaemia-reperfusion injury (Chambers *et al* 1985, Sanfey *et al* 1986, Granger *et al* 1986, Sarr *et al* 1987, Andrews *et al* 1994). These previous studies have shown that allopurinol is protective when the drug is administered prior to the ischaemic insult. While these studies have provided information regarding the pathophysiology of the local tissue injury, they are not relevant to the clinical situation, in which patients are usually presented after the ischaemic insult has occured. Using a model of ischaemia-reperfusion injury involving induction of cardiac arrest, followed by resuscitation in rats, Badylak *et al* (1986) found that administration of allopurinol at the onset of reperfusion (return of spontaneous circulation), increased long term survival, but in dogs with experimentally induced GDV, allopurinol treatment 15 minutes prior to gastric decompression did not increase survival (Badylak *et al* 1990). Further studies to assess the long term effects of allopurinol administration on the secondary complications of ischaemia-reperfusion injury are warranted. The preferred focus of these studies would be whether allopurinol ameliorates vascular injury in organs such as the lung.

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Chapter 5
Conclusions

Xanthine oxidase is an enzyme that plays an intimate role in the pathophysiology of the injury that occurs to an ischaemic organ following the resupply of oxygen. This injury is known as ischaemia-reperfusion injury and in dogs, and other species, it is associated with a high morbidity and mortality. The development of secondary complications, such as acute respiratory distress syndrome (ARDS), part of the systemic inflammatory response syndrome (SIRS), and disseminated intravascular coagulation (DIC), contribute to the mortality rate. This thesis has focused upon circulating xanthine oxidase as a possible mediator of these complications.

A spectrophotometric assay of uric acid production was developed for measuring xanthine oxidase activity in canine serum. This assay appears to be adequate for the detection of xanthine oxidase activity in canine serum, as it can detect as little as 11 milliunits of xanthine oxidase activity per litre (mU/l) of canine serum, when 400 microlitre aliquots are used. This is not as sensitive as the radioisotopic or ELISA techniques, but is less expensive and simpler to perform. The assay has a good reproducibility, with the coefficient of variation ranging from 2.1% to 12.8%. While a variation of less than 5% is desirable, this was not achieved in assays performed on different days, probably primarily due to day to day variation in the room temperature, which affects enzyme activity. This could be overcome in future studies by using a water bath to maintain a constant temperature for the reaction.

The activity of bovine milk xanthine oxidase, stored in canine serum at -20°C and -80°C, appeared to increase slightly over the first 7 days of storage, possibly due to ongoing conversion of xanthine dehydrogenase to xanthine oxidase. Thereafter, the xanthine oxidase activity appeared stable for 90 days. Therefore, serum samples can probably be stored for this period of time, without a significant decline in xanthine oxidase activity. Samples stored at +4°C were stable for 30 days, and this provides an alternative method of storage of serum prior to xanthine oxidase assay.

Serum xanthine oxidase activity in dogs has only been investigated once, over 30 years ago. The mean serum xanthine oxidase activity measured in that study, using only 3 dogs, was similar to that measured in this research project. A wide range of serum xanthine oxidase activities were measured in clinically "healthy" dogs. A variety of factors may affect the activity of this enzyme, but have largely remained uninvestigated in dogs. Exercise is one factor that does elevate the serum xanthine oxidase in horses, and may be worthy of investigation in dogs. Both of the non-parametric methods that were used to determine the reference limits, resulted in the determination of the same reference interval, of 0-204 mU/l. This reference interval only applies to the method and experimental conditions described in this thesis. The size of the reference sample population was too small to determine confidence intervals for the reference limits. Ideally, greater than 120 individuals should make up the reference sample population when non-parametric methods are used, and parametric methods could not be used in this study as the data did not have a Gaussian distribution. However, the reference values obtained were useful for direct comparison with the serum xanthine oxidase activities measured in sick dogs.

Prior to this research project, the serum xanthine oxidase activity had not been measured in dogs with ischaemic disorders. We determined that serum xanthine oxidase activity was significantly increased in these dogs, compared with "healthy" dogs, and that a further increase in serum xanthine oxidase activity usually occured after reperfusion. There was a trend for dogs with more severe clinical signs to have higher serum xanthine oxidase activities, and the lack of statistical significance of this finding may have been due to lack of statistical power. There was also a trend for dogs that died of respiratory failure, associated with ARDS, to have a higher serum xanthine oxidase activity than dogs that survived. Further studies would be helpful to determine the significance of these trends. Serum xanthine oxidase activity was not significantly different between dogs that survived or died, and therefore it does not appear to be useful as a test to predict the outcome of the disease.

The above findings are consistent with those of *in vitro* and *in vivo* ischaemia-reperfusion, and provide further evidence that xanthine oxidase may be one of the circulating mediators involved in the pathophysiology of ARDS, SIRS, and DIC. Allopurinol, a specific xanthine oxidase inhibitor, may be useful in clinical cases of ischaemia-reperfusion injury, to prevent or reduce the

severity of the secondary complications. Most studies have focussed upon the ability of allopurinol to attenuated local, acute tissue injury associated with ischaemia and reperfusion, but the drug appears to only be useful when given prior to the ischaemic insult. Future studies regarding the effect of allopurinol, administered after the ischaemic insult, or following reperfusion, on long term survival, and particularly the occurrence of secondary complications, such as vascular damage, are warranted. The latter studies would be preferable because the variability of severity in clinical cases with ischaemia-reperfusion injury would necessitate very large numbers of dogs to demonstrate statistical significance when using a crude indicator, such as survival, as the experimental end-point. Studies should also be performed to determine the length of time over which the serum xanthine oxidase activity is elevated following reperfusion.