ACTIONS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN SHEEP

by

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A thesis submitted for the degree of Doctor of Philosophy

in the Faculty of Veterinary Medicine

of the University of Glasgow

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August, 1993.

To my parents and Chris.

CONTENTS

ACKNOWLEDGEMENTS

This work was generously funded by the Agricultural and Food Research Council.

I would like to thank Dr. A. M. Nolan for her enthusiastic supervision of this work, and for her constant support and guidance.

I would like to thank Dr. Q. A. McKellar for allowing the research to be carried out in his department, and also the technical staff in the Department of Veterinary Pharmacology for their assistance. In particular, I would like to thank Mr P. Baxter, Mrs A. Galbraith and Mrs M. Michael for their assistance with the HPLC analysis, and Mr I. Gibson for his help in constructing the tourniquet.

I am indebted to Dr. G. Gettinby of the Department of Statistics and Modelling Science, University of Strathclyde, for his patience and encouragement whilst helping me with statistical methods.

I wish to express my thanks to Dr. G. Fishwick and Dr. J. Parkins, Department of Animal Husbandry, University of Glasgow, The Institute of Animal Physiology and Genetics Research, Roslin, Mr J. Blair, Barrhead and Mr I. Lamont, Lochwinnoch for allowing me access to their flocks of sheep.

I would like to thank Messrs. A. McFadyen, C. McFadyen, M. Williamson and the staff at Cochno Farm for managing the sheep used in the present study, and in addition, I would like to mention 'Meg' without whom, on occasion, the sheep may never have been gathered.

I am grateful to Mr A. Hudd and Mr B. Robb for skilfully constructing the thermal device used in these experiments, and to Mr. G. Baxter for his helpful advice with the mechanical device.

I would like to thank Ms. M. Ritchie, Ms. M. Thompson and Mr B. Wilson, who anaesthetised and operated on the sheep at IAPGR, for their unending cooperation and also Mr C. Blair for his assistance in taking the measurements.

DECLARATION

The contents of this thesis are the work of the author. The thesis has not been submitted previously to any university for the award of a degree. The following publications are based on the work contained in this thesis:

Welsh, E. W. , Baxter, P. , Nolan, A. M. 1992. Pharmacokinetics of carprofen administered intravenously to sheep. *Research in Veterinary Science.* **53**, 264-266.

Welsh, E. M. , McKellar, Q. A. , Nolan, A. M. 1993. The pharmacokinetics of flunixin meglumine in the sheep. *Journal of Veterinary Pharmacology and Therapeutics.* **16**, 181-188.

Welsh, E. W. , Gettinby, G. , Nolan, A. M. 1993. Comparison of a visual analogue scale and a numerical rating scale for assessment of lameness, using sheep as a model. *American Journal of Veterinary Research.* **54**, 976-983.

SUMMARY

Mechanical and thermal stimuli were used to determine threshold responses in normal experimental sheep which were familiar with both test procedures. The average control response threshold to noxious thermal and mechanical stimulation was 54.5°C and 3.2 Newtons (N), respectively. The effects of 2 non-steroidal anti-inflammatory drugs (NSAIDs), flunixin meglumine and carprofen, were investigated using the 2 test systems. Neither drug was shown to influence thresholds to noxious stimulation over a 6 hour period in normal sheep.

Thresholds to noxious mechanical stimulation were recorded from normal farm sheep which were unfamiliar with the testing procedure. The average response threshold in these sheep was significantly greater than that recorded from experimental sheep (mean, 4.9N), but fell over a period of 3 days to a similar level recorded from experimental sheep (mean, 3.1N). Thresholds to noxious mechanical stimulation in farm sheep which were unfamiliar with the testing procedure, and which had been suffering from footrot for a period of not less than 1 week, were not significantly different from normal farm sheep (mean, 4.7N). In addition, when these sheep were tested over a period of 3 days, thresholds did not fall in the manner described for normal sheep (mean, 4.6N). However, administration of flunixin meglumine, 1.0 mg/kg, IV, once daily for 3 days in sheep suffering from footrot caused a significant reduction in thresholds to noxious mechanical stimulation in sheep suffering from footrot on the third and fourth days after treatment had been initiated.

Thresholds to noxious mechanical and thermal stimulation were assessed in sheep undergoing anaesthesia and abdominal surgery, and the effects of flunixin meglumine, carprofen and buprenorphine, a partial opioid agonist, on thresholds investigated. Induction of anaesthesia was achieved by injection of thiopentone or ketamine, IV, and anaesthesia was maintained by administration of halothane in oxygen and nitrous oxide. After thiopentone induction, thermal and mechanical thresholds were not shown to change after a 20 minute period of general anaesthesia alone, and similarly, thresholds to noxious mechanical stimulation were not significantly different from control values after induction of anaesthesia with either thiopentone or ketamine in sheep which underwent abdominal surgery. However, after thiopentone induction, thresholds to noxious thermal stimulation were significantly lower than control values 45, 60 and 120 minutes post-operatively. Intra-operative injection of flunixin meglumine (1.0 mg/kg, IV) and buprenorphine (10 μg/kg, IV) prevented the development of post-operative thermal

hyperalgesia, while carprofen (4.0 mg/kg, IV), not only prevented the development of hyperalgesia in the immediate post-operative period, but also caused a significant increase in thresholds to noxious thermal stimulation 60 minutes post-operatively. Thresholds to noxious thermal stimulation were unchanged in the post-operative period in sheep anaesthetised with ketamine which subsequently had undergone abdominal surgery under halothane anaesthesia.

Thresholds to noxious mechanical stimulation were investigated during peripheral limb ischaemia, induced by application of a pneumatic tourniquet to the forelimb of sheep. During limb ischaemia, mechanical thresholds fell to below control values, and immediately prior to tourniquet deflation, were significantly lower than those recorded pre-inflation (3.1N *vs* 1.7N). Injection of flunixin meglumine (1.0 mg/kg, IV) and carprofen (0.7 mg/kg, IV) 1 hour prior to tourniquet inflation attenuated the fall in mechanical thresholds recorded after injection of saline (5 ml (0.9 %), IV) 1 hour before inflation. Similarly, administration of fentanyl (5 μg/kg, IV), a μ-opioid agonist, prevented the reduction in thresholds observed during tourniquet inflation.

Thresholds to noxious mechanical and thermal stimulation were assessed for a period of 120 minutes after intradermal injection of saline (0.9 %, 100 μl) or the irritant carrageenan (0.0625 %, 100 μl). Flunixin meglumine (2.0 mg/kg, IV), carprofen (4.0 mg/kg, IV) or saline (5 ml (0.9 %), IV) was administered at 120 minutes, and subsequently, thresholds to noxious stimulation were investigated for a further 4 hours. Intradermal injection of carrageenan did not cause a clearly defined change in thresholds to noxious mechanical stimulation, but thresholds to thermal stimulation were significantly lower than control values 120 minutes after injection of the irritant (group mean, 52.4°C *vs* 49.3°C). Thresholds to noxious thermal stimulation remained significantly lower than control values after administration of saline, IV, for a further 60 minutes. However, after administration of either NSAID, thresholds were no longer significantly different from control values.

Pharmacokinetic analyses of plasma levels of flunixin meglumine and carprofen (0.7 and 4.0 mg/kg) following intravenous injection in sheep was carried out. The decline of flunixin meglumine in plasma was best described by a tri-exponential equation, after injection of both 1.0 and 2.0 mg/kg, with elimination half-lives of 221.7 and 205.8 minutes, respectively. The decline of carprofen in plasma was best described by a bi-exponential equation, after injection of both 0.7 and 4.0 mg/kg, with elimination half-lives of 25.8 and 32.3 hours, respectively.

A comparison of 2 subjective rating scales, an numerical rating scale (NRS) and a visual analogue scale (VAS), for rating lameness in sheep was made. Both scales were shown to be reproducible and repeatable, but the VAS was more sensitive than the NRS and did not force observers to group unlike data. It was demonstrated that although reproducibility of both the VAS and NRS was poor when an untrained observer was compared with a trained observer, the repeatability of both scales was good for untrained as well as trained observers. The VAS was used to score a group of sheep for lameness and to assess the response to flunixin meglumine (1.0 or 2.0 mg/kg, IV) over a 6 hour period after injection. No significant differences were shown between the 2 different dose rates used in the study, nor were there any significant differences within either group. However, injection of flunixin meglumine caused a reduction in lameness in >80% of sheep administered the lower dose at some time after injection, while >70 % of sheep administered the higher dose rate showed a similar reduction.

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ABBREVIATIONS

aufs	absorbance units full scale
CNS	central nervous system
δ	delta
e. g.	for example
et al.	and others
etc.	*et cetera*
Fig.	figure
g	gravity, 10^{-11} N.m/s^2
Hg	mercury
HPLC	high performance liquid chromatography
hr	hours
i. e.	that is
ICV	intracerebroventricular
ID	intradermal
IM	intramuscular
iu	international units
IV	intravenous
kg	kilograms
kPa	kiloPascal
l	litres
λ	wavelength

M	molar
μ	mu
μg	micrograms
mg	milligrams
min	minutes
μl	microlitres
mm	millimeters
mV	millivolts
ng	nanograms
nm	nanometers
No.	number
obs	observed values
p	probability
pH	negative logarithm of hydrogen ion concentration
PMNL	polymorphonuclear leucocytes
R	*rectus*
r	Pearson linear correlation coefficient
S	*sinister*
SEM	standard error of the mean
spp.	bacterial species
uv	ultraviolet
vide infra	see below

vide supra	see above
vs	*versus*
ºC / °C	degrees centigrade

CHAPTER 1

GENERAL INTRODUCTION

Pain is a subjective experience, the definition and description of which is unique to the individual that experiences the painful stimulus, and it should be accepted that pain is what the subject says is painful (Beecher, 1957). Humans have the luxury of language and can attempt to convey to a second party the temporal, spatial and affective aspects of the sensation which they perceive as painful. An observer of human pain also may notice alterations in the normal physiological responses such as an increase in blood pressure, heart rate, respiratory rate or guarding of the painful area. The evaluation of pain in animals becomes more challenging where assessments of pain rely exclusively on observation of animal behaviour and of physiological changes.

The ability to quantify and qualify pain is a pre-requisite to the assessment of the analgesic efficacy of drugs both experimentally and clinically, and requires a knowledge of the neurophysiological and neuroanatomical events which occur during nociception.

1.1 Mechanisms of noxious information processing

The anatomical pathways and physiological mechanisms of transmission of noxious information from the periphery to the central nervous system (CNS) have been reviewed in detail by Zimmerman (1976) and Yaksh and Hammond (1982). Briefly, sensory information is received by a variety of end organs, and it is generally accepted that those responsible for the sensation of pain are free nerve endings which are located widely throughout the tissues of the body. The free nerve endings subserving detection of pain are referred to as nociceptors, and respond to a variety of noxious stimuli including high intensity mechanical and thermal stimuli and also to noxious chemical stimulation by either endogenous or exogenous substances. Noxious stimulation causes impulses in one of 2 main types of primary afferent nerve fibre *i. e.* non-myelinated C-fibres or Aδ-fibres, which are the slowest conducting myelinated fibres. The cell bodies of the primary afferent fibres are located in the dorsal root ganglia and the fibres enter the dorsal horn of the spinal cord within the dorsal roots. The grey matter of the spinal cord is divided into a series of numbered laminae (Rexed, 1952). Within the cord the primary afferent fibres synapse with the neurones of the cord. The faster conducting Aδ-fibres synapse mainly in laminae I and V, whereas the slower conducting C-fibres synapse primarily in lamina II, or the substantia gelatinosa. Conscious perception of pain requires the onward transmission of noxious information from the spinal cord to the higher centres, although the response to the noxious stimulus may involve activation of a local spinal reflex. Fibres carrying nociceptive information ascend from the dorsal horn of the spinal cord, both contra- and ipsilaterally, in the ventrolateral columns of the spinal cord, to 3 main areas of the brain, the reticular formation (spinoreticular fibres or anterolateral tract), periaqueductal grey matter (PAG) (spinomesencephalic or spinobulbar fibres) and to the medial and ventrobasal thalamus

(spinothalamic fibres). The sensory nucleus of the spinal tract of the trigeminal nerve, is the most caudal segment of the sensory nucleus of the trigeminal nerve, and is continuous with the dorsal horn of the spinal cord. It receives the central projections of all neurones carrying nociceptive and thermal information from the head region, which travel to the reticular formation and the ventroposterior thalamus.

Stimulation of various supraspinal structures in the medulla and pons, including the nucleus raphe magnus, nucleus reticularis paragigantocellularis and locus coeruleus, in the midbrain *e. g.* the PAG, and also in the fore brain *e. g.* lateral hypothalamus and ventrobasal thalamus, can cause analgesia and inhibit transmission in nociceptive neurones (Carstens, 1983). These actions are mediated by activation of serotonergic and noradrenergic pathways which have a mainly depressant action on spinal cord neurones (Belcher *et al.* , 1978), although endogenous opioid systems have been implicated (Finley *et al.* , 1981; Woolf and Wall, 1983). The descending pathways are one of the important mechanisms in the 'Gate Theory of Pain' proposed by Melzack and Wall (1965), where they suggested that descending efferents from supraspinal centres modulated transmission of noxious information. Moreover, it was suggested that lamina II neurones acted to regulate transmission between primary afferent fibres and ascending tracts, *via* a network of interneurones. Within this network, inhibitory interneurones are activated by both descending inhibitory influences and non-noxious primary afferent input *e. g.* from mechanoreceptors *via* large diameter Aβ-fibres, whereas nociceptive afferent fibres activated facilitatory interneurones. Finally, the somatosensory cortex receives input from the 3 main ascending tracts already mentioned, and presumably mediates the sensory-discriminative aspects of pain.

1.2 Production and assessment of nociceptive stimuli

The pain threshold is the intensity of stimulation which is first perceived as pain and generally is considered to be approximately the same in humans and animals (Vierck, 1976). The intensity of stimulation at which the subject can no longer tolerate the pain is known as the pain tolerance threshold (Vierck, 1976). There are 4 main stimulus types used to induce experimental pain, mechanical, thermal, chemical and electrical. These stimuli may be used in 2 main ways to elicit these responses, either fixed stimulation intensity with different stimulation duration, or fixed stimulation duration with variable stimulus intensity. When these tests are applied in the normal animal they elicit physiological pain *i. e.* they result in a transient, localized and stimulus specific reaction (Woolf, 1989), but they may also be used to induce pathological pain *e. g.* thermal, chemical or electrical 'burns', adjuvant-induced arthritis or experimental surgery after which stimulation results in a diffuse and persistent reaction with spatial spread of pain to uninjured tissues. In addition, reduction of thresholds

to painful stimuli (allodynia) and an increase in response to the stimulus (hyperalgesia) may be observed (Woolf, 1989; 1991). However, it is important to note that the latter 2 terms frequently are used interchangeably. Clinical pain is found to have many of the features of pathological pain. The source of clinical pain is not always apparent, although direct trauma, and many disease states, result in the development of acute or chronic pain.

Although recent developments in cell culture have aided investigation of cellular mechanisms of pain and hyperalgesia (Ortmann and Perkins, 1977; Baccaglini and Hogan, 1983; McGuirk and Dolphin, 1992; Nicol *et al.* , 1992), experimental pain allows evaluation of new analgesic agents and is used extensively in the investigation of the mechanisms involved in the induction of hyperalgesia and persistent pain. However, it is important that every effort is made to minimise distress to experimental animals and to operate within ethical guidelines. Beecher (1957) recommended that the following points should be taken into consideration when inducing experimental pain: the chosen stimulus should be applied to a part of the body where measured responses are associated with pain; the stimulus should result in little tissue damage at the pain threshold level, and the hazard to the subject be small at highest intensity; there should be a relationship between the intensity of stimulus and the intensity of pain experienced; repeated determinations using the same stimulus should not affect the quantifiable response; the stimulus should result in a clear end point; the stimulus should allow for small alterations in threshold to be detected and should allow for detection of different doses of analgesic, and finally, it should be applicable in both man and animals. Since these recommendations were made, the subject has been reviewed extensively and further recommendations added, specifically with reference to induction of chronic pain (Zimmerman, 1983; Dubner, 1987; Loew, 1987).

1.2.1 Mechanical methods

Von Frey (1897) first described the use of hairs of varying diameter and length to bend the cutaneous epithelium in man to evoke pain. Horse hairs were attached to a lever, and the weight required to bend the hair and evoke a painful response was evaluated. The method was limited after the administration of analgesics because the operational weight range was narrow. Various modifications of the original system have been tried in an attempt to increase the effective range of the system *e. g.* by applying the stimulus to more sensitive skin regions (Seevers and Pfeiffer, 1936). Pressure applied to the tail of animals was used in the 1920s (Eddy, 1928; Franklin and Abbott, 1989), and attempted escape or vocalization were used as the end-points. However, this method was not found to be reliable in all species (Smith, 1938), and occasionally, tissue damage was evident after repeated use (Friend and Harris, 1948), which influenced thresholds determined subsequently.

Green and Young (1951) described apparatus which applied a steadily increasing pressure to the tail of the rat and which was quantifiable. They showed that application of a pressure 4 times greater than threshold pressure did not result in tissue damage, and recommended such a pressure as maximal. A modification of this system, where pain threshold in an inflamed rat paw is assessed (Randall and Selitto, 1957), currently is used widely in analgesic drug testing. In the domestic species, various systems of assessment of thresholds to noxious mechanical stimulation have been described (Pippi and Lumb, 1979; Nolan *et al.* , 1987a; Hamlin *et al.* , 1988). Pressure also may be used to produce visceral pain, and distension of the oesophagus, common bile duct and intestine have been investigated in humans (Chapman *et al.* , 1949). Similarly, in the domestic species distension of the caecum or colon has been used as a method of investigating thresholds to noxious stimuli (Pippi *et al*, 1979; Sawyer and Rech, 1987). Ischaemic muscle pain also has been induced by applying pressure around an extremity to occlude blood flow (Hewer and Keele, 1948).

1.2.2 Thermal Methods

Radiant Heat

Heat applied to the skin, initially by contact through hot water or hot objects, was used for many years, but was limited by the concurrent sensations of touch and pressure evoked at the same time (Beecher, 1957). Oppel and Hardy (1937) demonstrated that radiant heat could be used to allow quantification of response temperature in man and in 1940, Hardy and colleagues used fixed duration, variable intensity radiant heat to measure thresholds to noxious thermal stimulation in man. In the latter study, the radiant energy from a 500 or 1000 watt projection lamp was focused for a defined period of time onto an area of skin which had been blackened. An electrically timed shutter was used to control exposure, and a rheostat to control the current delivered to the lamp which varied the intensity of radiation. In an attempt to limit experimental variation resulting from the degree of skin blackening achieved, a colour filter was employed by Flodmark and Rammer (1945). Variations from the original method described by Hardy *et al.* (1940) in humans, including fixed intensity variable duration, has been used in many different species, including rats (D'Amour and Smith, 1941), dogs (Andrews and Workman, 1941), guinea pigs (Winder *et al.* , 1946), cats (Booth, 1954) and horses (Pippi *et al.* , 1979; Kamerling *et al.* , 1985; Schatzmann *et al.* , 1990).

Conducted heat

The 2 most widely used alternatives to the radiant heat method of Hardy *et al.* (1940) are the hot-plate test (Woolfe and McDonald, 1944) and the tail immersion test (Janssen *et al.* , 1963) which employ conduction of heat. In the hot-plate test (Woolfe and McDonald, 1944), a metal plate is heated to a pre-determined level and mice dropped onto the plate. The latency to response is measured and responses included shaking and licking the paws and kicking. The use of hot-plates which increase in temperature over time also has been described, where threshold temperature rather than response latency is recorded (Oden and Oden, 1982; Hunskaar *et al.* , 1986a). The second test using conducted heat is the tail immersion test (Janssen *et al.* , 1963), where the rat's tail is submerged in water heated to a pre-set temperature and response latency measured. Thresholds to noxious thermal stimulation also have been tested using conducted heat in the larger domestic species, where a heating element is placed in contact with the animals skin and either the response temperature, or time to response is measured (Nolan *et al.* , 1987a; Schatzmann *et al.* , 1990).

Threshold to noxious thermal stimulation also may be assessed by investigating the response to cooling (Kunkle, 1949), although currently this method is not frequently employed in assessments of analgesic efficacy.

1.2.3 Electrical methods

Many tests using electric impulses to activate nociceptors have been described, but currently there are only a limited number of methods used for the evaluation of nociceptive responses. The tooth-pulp stimulation test relies upon the reflexive action of opening the jaw, observed after electrical stimulation of the tooth, or recording the electromyographic response from the jaw muscles (Chapman *et al.* , 1985). Chewing and licking, and gross escape behaviour also have been noted (Ha *et al.* , 1978; Mason *et al.* , 1985). This test has been described in many species, including humans, rats, monkeys, dogs, cats, rabbits, guinea pigs (Franklin and Abbott, 1989) and horses (Brunson *et al.* , 1985).

The flinch-jump test was first described by Evans (1961), and measures the threshold to an electrical shock transmitted through the floor of a test chamber at which the rat either flinches or jumps, characterised by elevation of 1 or more paws from the floor. Electrical shocks also may be used to induce vocalization in mice, as well as other rodents (Nilsen, 1961). Electrical shocks are administered *via* cutaneous electrodes or clips positioned at the base of the tail, and the threshold is the lowest shock intensity that elicits a squeak.

1.2.4 Chemical methods

Noxious stimulation resulting from the injection or application of chemical substances differs from those methods already mentioned, because the animal cannot limit the extent of the aggravation once induced. In 1951, Armstrong and colleagues described the production of a blister base, by removing the epidermis from a blister induced by cantharidin, and assessed the response to application of chemicals to the base by asking subjects to squeeze a pressure bulb. Chemical methods commonly used in the assessment of nociceptive responses currently, include the writhing response observed in rodents after intraperitoneal administration of irritants, first described by Vander Wende and Margolin (1956), the formalin test (Dubuisson and Dennis, 1977) and the Randall-Selitto test (Randall and Selitto, 1957), which combines chemically-induced inflammation and evaluation of thresholds to noxious mechanical stimulation. Chemically-induced arthritis also is used in the assessment of potential analgesics (for review see Otterness and Bliven, 1985). These methods are discussed in more detail in chapter 4.

1.3 Responses to noxious stimuli

Responses to noxious stimulation in nature have a protective role aimed at avoiding or limiting tissue damage, and may be classified into the following categories: simple reflexes such as limb withdrawal; unlearned behaviour such as vocalization and learned behaviour such as escape detection (Bonica, 1992).

1.3.1 Animals

In animals, the simple reflex behaviours are assessed either by measuring the response latency or the intensity at which the response occurs *e. g.* the tail flick reflex in the rat, (D'Amour and Smith, 1941), where the end point is when the tail is flicked. Further simple reflex behaviours include limb withdrawal tests (Randall and Selitto, 1957; Pippi and Lumb, 1979; Nolan *et al.* , 1987a; Hamlin *et al.* , 1988), skin flinch (Evans, 1961; Kamerling *et al.* , 1985) and jaw opening reflexes (Mitchell, 1964). Interpretation of data from simple reflex behaviours can be complicated by alterations in motor function as well as sensory processing, and do not consider the affective component of pain. Organized unlearned behaviour includes paw licking or face rubbing (Woolfe and MacDonald, 1944; Chapman *et al.* , 1985), writhing responses (Siegmund *et al.* , 1957) and vocalization (Eddy, 1928), and probably involves affective responses. Learned responses also may be used in threshold assessment *i. e.* shock-titration. These responses require the animal to exhibit learned behaviour to avoid noxious stimuli and escape latency is recorded (Chapman *et al.* , 1985).

1.3.2 Man

Many of the simple reflex behaviours which are exhibited by animals in response to noxious stimuli are not demonstrated in humans. However, in psychophysical experiments, the human subject may be asked to convey their impression of the intensity of the applied stimulus verbally or using rating scales. Thus, the end-point of many test systems in humans is taken when the subject reports that the pain threshold, or tolerance, has been reached *e. g.* radiant thermal stimulation of the forehead (Hardy *et al.* , 1952) and cold water stimulation (Wolf and Hardy, 1941). Using rating scales the subject is asked to complete a structured categorized scale according to the severity of pain they are experiencing. The scales used include unidimensional scales *e. g.* visual analogue scale, numerical rating scale (see chapter 6), and multidimensional scales *e. g.* McGill Pain Questionnaire (Melzack, 1975). However, it should be noted that these systems can be sensitive to placebo effects, expectancy and other psychological variables (Chapman *et al.* , 1985). Various other psychophysical and physiological methods have been employed in human pain estimation, and are reviewed by Chapman *et al.* (1985).

1.4 Analgesics

There are many drugs which have been shown to exert analgesic effects, both experimentally and clinically, and some of them will be reviewed briefly. Those used most commonly include the non-steroidal anti-inflammatory drugs (NSAIDs), opioids and α_2-adrenoreceptor agonists. Various other agents have also been shown to provide analgesia but will not be considered here. These include the local anaesthetic agents, ketamine, benzodiazepines and muscarinic agonists such as acetylcholine (de Jong and Wagman, 1963; Owen *et al.* , 1987; Sawynok, 1987; Maurset *et al.* , 1989; Bartolini *et al.* , 1992).

1.4.1 Opioids

Opioid drugs exert their analgesic actions by binding to specific opioid receptors, including mu (μ), delta (δ) and kappa (κ), in the brain and spinal cord, and there are a variety of endogenous ligands which act at these receptors (Martin *et al.* , 1976; Lord *et al.* , 1977; Dubner and Bennett, 1983). Opioid receptors also have been identified in the periphery and opioid drugs have been shown to have peripheral analgesic effects (Ferreira and Nakamura, 1979a; Fields *et al.* , 1980; Joris *et al.* , 1987). Opioid drugs, which may be full agonists, antagonists, have partial agonist activity or act as agonists at one receptor while exerting antagonistic effects at another (mixed agonists/antagonists), have different binding selectivities on different receptor subtypes. The opioids are used widely for the control of moderate to severe pain, particularly in the peri-operative period (Mitchell and Smith, 1989).

Unfortunately, there are a variety of side-effects associated with the use of these drugs, including tolerance, dependence, respiratory depression and cardiovascular effects (see Martin, 1984).

1.4.2 α_2-adrenoreceptor agonists

The α_2-adrenoreceptor agonists *e. g.* clonidine, xylazine and detomidine, have been shown to be potent analgesic agents in a variety of species (Schmitt *et al.* , 1974; Fielding *et al.*, 1978; Nolan *et al.* , 1987a; 1987b; Chambers *et al.* , 1993a; Kyles *et al.* , 1993), and the analgesic actions of these drugs is mediated, at least partially, at the level of the spinal cord (Kyles *et al.* , 1993). Injection of the α_2-adrenoreceptor agonists can cause various side-effects, as described for the opioids, including cardiovascular and respiratory depression (see Greene and Thurmon, 1988).

1.4.3 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs are those substances, other than steroids, which suppress one or more components of the inflammatory response (Lees and Higgins, 1985). This definition encompasses many non-specific agents and consequently, in this thesis, the term NSAID will be used to refer to those drugs which inhibit some component of the enzyme systems involved in the metabolism of arachidonic acid. The NSAIDs used clinically are generally weak organic acids and possess analgesic and anti-pyretic properties in addition to their anti-inflammatory actions.

Approximately 8 % of people in the United States of America have a rheumatic symptom at any one time (Brooks and Day, 1991), and this is reflected in the number of prescriptions written for NSAIDs in human medicine. More recently, these drugs have been used for the control of post-operative pain, either on their own or in conjunction with more traditional therapy (Mitchell and Smith, 1989). In veterinary medicine, the NSAIDs are widely used for a variety of conditions, and in many species the NSAIDs are the only drugs licensed for use which possess analgesic properties.

The NSAIDs used clinically are weak organic acids and may be further subdivided into 2 groups, the carboxylic acids and the enolic acids. The salicylates (*e. g.* acetylsalicylic acid, sodium salicylate), indolines (*e. g.* indomethacin, zomepirac, diclofenac), propionic acids (*e. g.* ibuprofen, naproxen, ketoprofen, carprofen), quinolines (*e. g.* cinchophen), aminonicotinic acids (*e. g.* flunixin meglumine) and anthranilic acids (meclofenamic acid, tolfenamic acid) are all carboxylic acids, while the pyrazolones (*e. g.* phenylbutazone, dipyrone) and oxicams (*e. g.* piroxicam and miloxicam) may be classified as enolic acids. A

comprehensive review of the acidic NSAIDs has been made (Lombardino, 1985), and only flunixin meglumine and carprofen will be considered in greater detail.

Flunixin meglumine is a methyl homologue of niflumic acid, and relatively little has been published in the human literature relating to this drug (Ciofalo *et al.*, 1975; 1977; Zederfeldt *et al.*, 1977; Sunshine *et al.*, 1984). However, flunixin is licensed for use in dogs, horses and cattle in the United Kingdom, and many experimental and clinical studies have reported the use of this agent. The pharmacokinetics of flunixin have been investigated in several of the domestic species (see chapter 5), and it is a potent inhibitor of the generation of metabolites of arachidonic acid metabolism (Semrad *et al.*, 1985; Higgins *et al.*, 1986; McKellar *et al.*, 1989; Lees and Taylor, 1991; McKellar *et al.*, 1991a; Soma *et al.*, 1992). Its use has been described for the control of pain and inflammation associated with musculoskeletal disorders (Houdeshell and Hennessey, 1977; McKellar *et al.*, 1991b), respiratory conditions (Selman *et al.*, 1986), mastitis (Anderson *et al.*, 1986; Lohuis *et al.*, 1989), colic (Vernimb and Hennessey, 1977), post-operative pain (Reid and Nolan, 1991) and endotoxic shock (Moore *et al.*, 1981; Hardie *et al.*, 1983; Hardie *et al.*, 1985a) in the domestic species.

Carprofen, a propionic acid derivative, has more recently been launched onto the veterinary market in the United Kingdom, although it has been available for humans in other European countries for some time. Carprofen is presented in solution as a racemic mixture, and the pharmacokinetics of the racemate, and also of the enantiomers have been investigated in several of the domestic species (see chapter 5). Unlike flunixin, carprofen is a poor inhibitor of cyclooxygenase, an important enzyme for the metabolism of arachidonic acid, although it is a potent anti-inflammatory agent (Randall and Baruth, 1976; Strub *et al.*, 1982). In addition, carprofen has no activity against another important enzyme for the metabolism of arachidonic acid, lipoxygenase, but it has been shown to have an effect on one of the enzymes regulating the release of arachidonic acid from its membrane bound site (Hope and Welton, 1983). Clinical studies investigating the use of carprofen are limited, but McKellar *et al.* (1991b) reported that carprofen was a useful anti-inflammatory and analgesic agent in degenerative joint disease in dogs. Moreover, it has been used successfully in the treatment of a variety of rheumatic complaints, and for the control of acute pain in man (Feldmeier, 1982; Klein, 1982; Standel, 1982).

1.4.3.1 The pharmacology of the NSAIDs with reference to their anti-nociceptive actions

The pharmacology of the NSAIDs cannot be discussed without a knowledge of the metabolic pathways at which this group of drugs have been shown to act. Consequently, the metabolism of the polyunsaturated fatty acid, arachidonic acid, will be reviewed.

1.4.3.1.1 Arachidonic acid metabolism

Ulf von Euler (1936) reported the isolation of an acidic polar lipid from human semen which he called prostaglandin, and which later was shown to be a combination of several different lipids, including prostaglandin E_1 and prostaglandin $F_{1\alpha}$ (Bergstrom and Sjovall, 1960). Bergstrom and colleagues (1963). later reported the isolation of several other stable prostaglandins, and shortly afterwards, it was demonstrated that the mono-, bis- and tri-enoic prostaglandins were formed from the polyunsaturated fatty acids, dihomo-γ-linolenic acid (8, 11, 14-eicosatrienoic acid), arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid) and timnodonic acid (5, 8, 11, 14, 17-eicosapentaenoic acid), respectively (Bergstrom *et al.*, 1964; Van Dorp *et al.*, 1964). Piper and Vane (1969) detected the release of a labile substance during anaphylaxis in isolated perfused guinea pig lungs, half-life of approximately 2 minutes, which they called rabbit aorta contracting substance (RCS), and they reported that its release was inhibited by the aspirin-like drugs. Although release of RCS from lungs in *in vitro* preparations could be demonstrated after challenge with a variety of endogenous and exogenous substances, including arachidonic acid (Palmer *et al.*, 1973), this substance did not match any of the known prostaglandins. Vane (1971) suggested that RCS was an unstable intermediate in the biosynthesis of prostaglandins, the possibility of which had been suggested previously by Samuelsson (1965). The presence of an unstable intermediate product was confirmed, and identified as PGH_2 (15-hydroperoxy-9, 11-endoperoxide) by Hamberg and Samuelsson (1973), and other workers reported the presence of a second intermediate in the same year, referred to as PGG_2 (15-hydroxy-9, 11-endoperoxide) (Nugteren and Hazelhoff, 1973), and they suggested that these unstable products were the same substances which had been referred to previously as RCS.

The unstable intermediate products, PGG_2 and PGH_2, or the cyclic endoperoxides, are the precursors for a wide variety of prostanoid products, including PGD, PGE and PGF (Fig. 1.1). Hamberg and co-workers (1975) reported the presence of an unstable non-prostanoid substance generated from the cyclic endoperoxides during platelet aggregation, which was identified as thromboxane A_2 (TXA_2). Thus, this product also had been

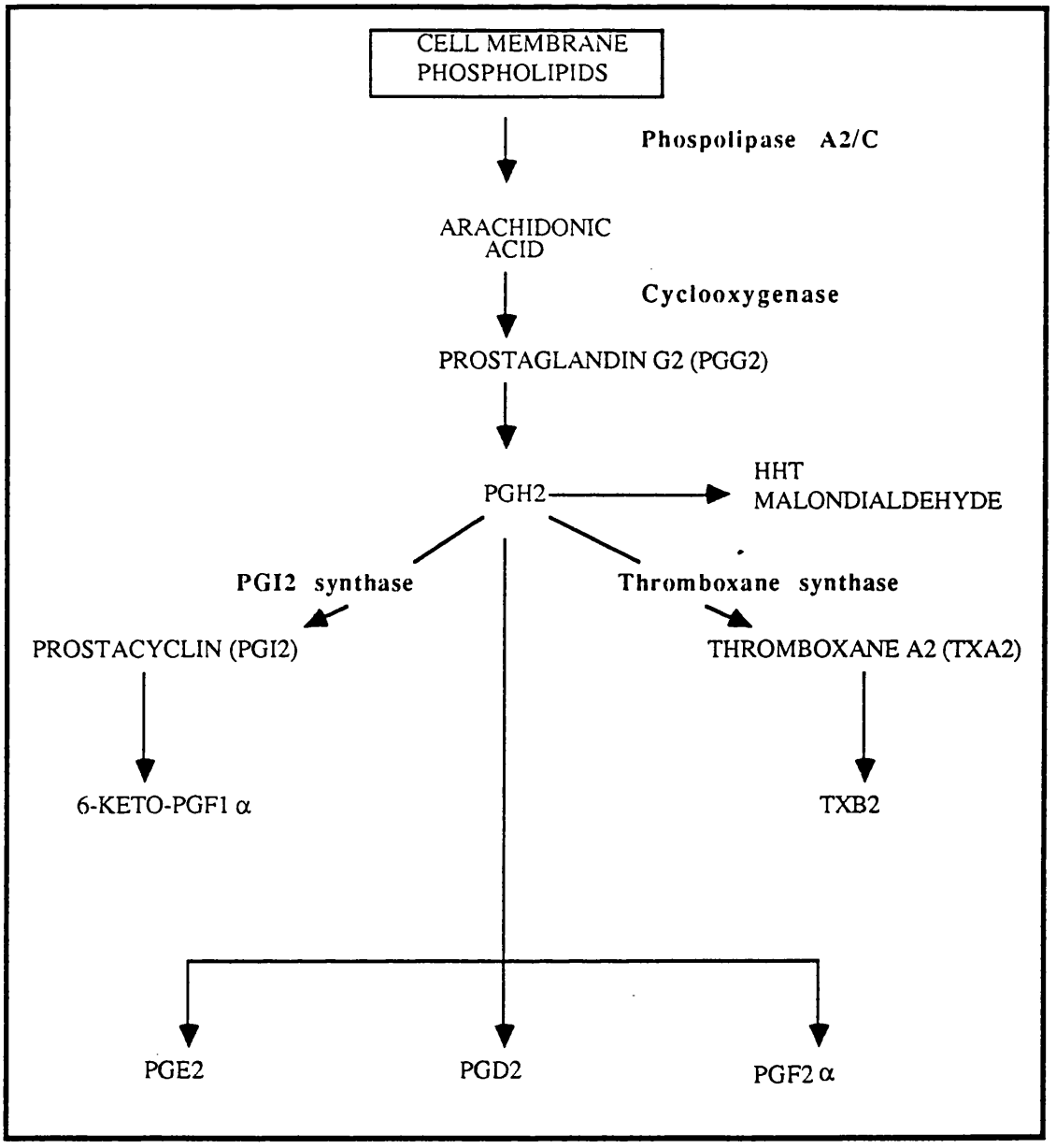

Figure 1.1. Simplified representation of the biosynthesis of prostaglandins (PG) and thromboxanes (TX) from arachidonic acid.

HHT- hydroxyheptadecanoic acid

partly responsible for the activity of RCS. Shortly afterwards, a novel prostanoid, prostacyclin, or PGI_2, was described, which was again unstable (Moncada *et al.* , 1976), and which was later shown to be the intermediate product in the formation of the stable prostanoid product, 6-keto-$PGF_{1\alpha}$ (Johnson *et al.* , 1976). These discoveries, coupled with the earlier characterisation of a second metabolic pathway for arachidonic acid leading to the production of 12-hydroperoxyeicosatetraenoic acid (12-HPETE), an unstable intermediate, and 12-hydroxyeicosatetraenoic acid (12-HETE), indicated that the generation of prostaglandins was only part of the story of arachidonic acid metabolism (Hamberg and Samuelsson, 1974) (Fig. 1.2). Moreover, in 1976, Borgeat and colleagues described the production of 5-hydroperoxyeicosatetraenoic acid (5-HPETE), an unstable intermediate product, and 3 years later, 5-hydroxyeicosatetraenoic acid (5-HETE) was discovered (Borgeat and Samuelsson, 1979), the metabolic product of which was an unstable epoxide of arachidonic acid, later named leukotriene A_4 (LTA_4) (Samuelsson *et al.* , 1979). Leukotriene A_4 also was unstable, and was transformed into LTB_4 by the action of LTA hydrolase, or alternatively was conjugated with glutathione to form LTC_4 (Murphy *et al.* , 1979; Samuelsson *et al.* , 1979). Finally, it was demonstrated that the formation of the remaining leukotrienes, LTD_4, LTE_4 and LTF_4, occured in sequence from LTC_4 (see Piper, 1984; Samuelsson *et al.* , 1987) (Fig. 1.2). Leukotriene C_4, D_4 and E_4 now are considered to represent the 'slow reacting substances of anaphylaxis', initially described in 1938 by Feldberg and Kellaway. The prostaglandins, thromboxanes and leukotrienes, are collectively referred to as the eicosanoids, 20 carbon derivatives of polyunsaturated fatty acids such as arachidonic acid.

Arachidonic acid, the most common precursor of the naturally occurring prostaglandins, thromboxanes and leukotrienes, may be obtained directly from the diet or by anabolic desaturation and chain elongation from dietary linoleic acid (Moncada and Vane, 1979). Within the body, it is transported bound to albumin, and is incorporated into the phospholipids of cell membranes, as well as other subcellular structures, of all tissues (Moncada and Vane, 1979). Arachidonic acid is found mainly in esterified form in the *sn*-2 position of membrane phospholipids, which suggests that phospholipase A_2 (PLA_2) is probably responsible for the hydrolization and release of arachidonate, forming the rate limiting step in the production of the eicosanoids (Flower and Blackwell, 1976). Burch and Axelrod (1987) demonstrated that bradykinin, by interacting with plasma membrane-bound receptors coupled to guanine nucleotide-binding regulatory proteins, caused direct activation of PLA_2, leading to the production of PGE_2. Phospholipase C also may be involved in the generation of free arachidonate within the body (Lapetina, 1982), as may

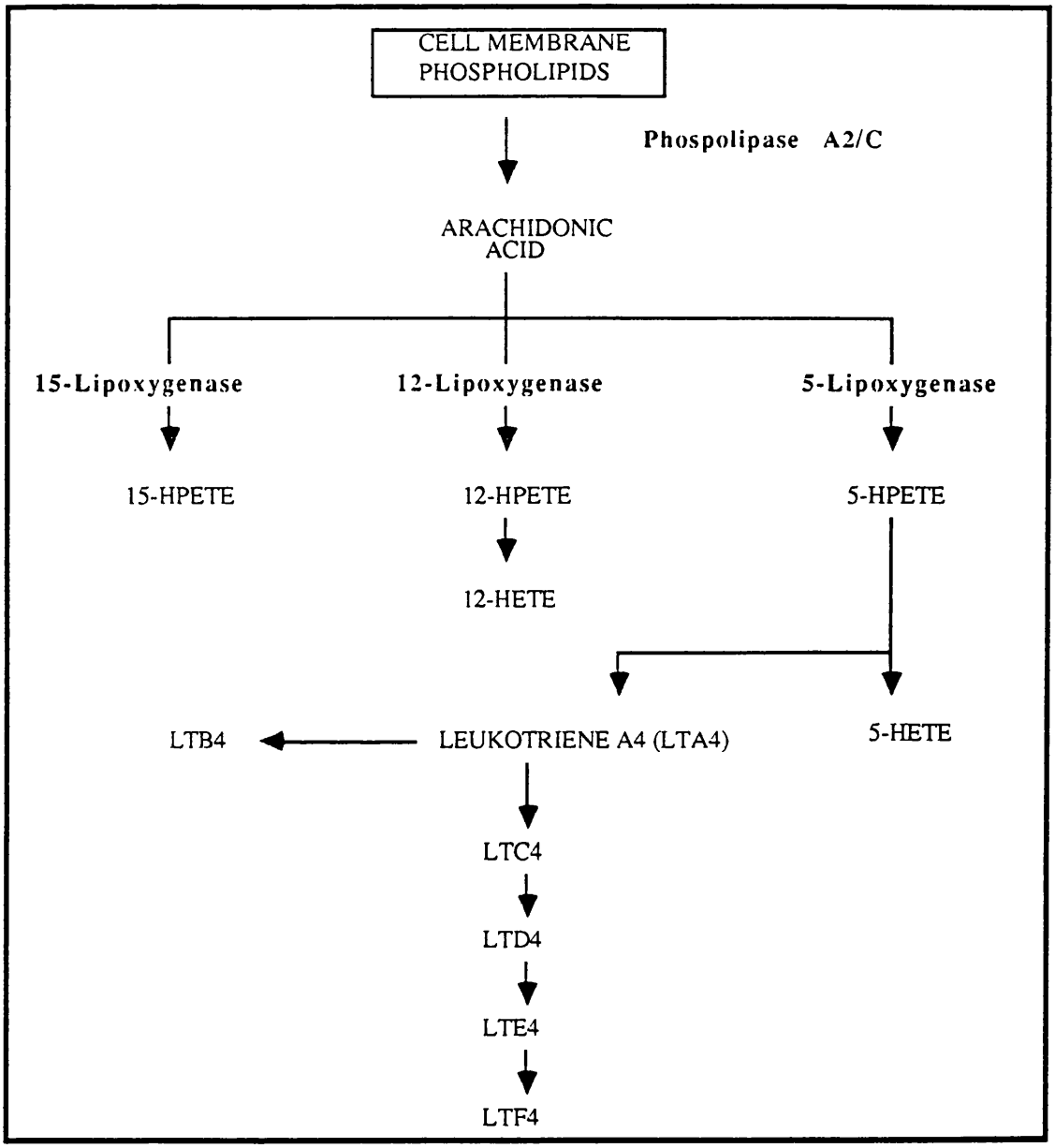

Figure 1.2. Simplified representation of the metabolism of arachidonic acid by the lipoxygenase group of enzymes.

HPETE- hydroperoxyeicosatetraenoic acid

HETE- hydroxyeicosatetraenoic acid

LT- leukotriene

the diacyglycerol lipase enzymes (Okazaki *et al.* , 1981). A variety of stimuli have been shown to result in increased prostaglandin production, and thus activation of the phospholipase enzymes, including mechanical, physical, hormonal, nervous and immunological stimuli (Granstrom, 1983). There are 2 main forms of PLA_2, extracellular and intracellular forms, and the regulation and inhibition of PLA_2 enzymes was recently reviewed (Glaser *et al.* , 1993).

Once liberated from cell membrane phospholipid, free arachidonate is either immediately re-incorporated into the cell membrane or metabolized by one of 2 enzyme pathways, either *via* the prostaglandin endoperoxide synthetase system, or *via* the lipoxygenase system (Higgins, 1985; Toh *et al.* , 1992). Prostaglandin endoperoxide synthetase, hereafter referred to as the cyclooxygenase enzyme system (PGH synthase), is a bifunctional enzyme with both fatty acid cyclooxygenase activity (arachidonic acid to PGG_2), and prostaglandin hydroperoxidase activity (PGG_2 to PGH_2) (*vide supra*). Immunocytochemical studies have located these enzymes to the endoplasmic reticulum and nuclear membrane within cultured fibroblasts (Rollins and Smith, 1980), and recently it was demonstrated that the cyclooxygenase enzyme is encoded by at least 2 genes that are expressed, and differentially regulated, in a variety of cell types (Hla and Neilson, 1992), supporting previous proposals that isoenzymes of cyclooxygenase existed (Smith and Lands, 1972; Flower and Vane, 1973; Lin *et al.* , 1989). The lipoxygenase system is comprised of a series of lipoxygenases, that peroxidize arachidonic acid, by incorporation of one oxygen molecule, at different carbon atoms *e. g.* 5-, 12- and 15-lipoxygenase resulting in the formation of the unstable hydroperoxides described above.

1.4.3.1.2 Inhibition of eicosanoid biosynthesis by NSAIDs

Several mechanisms of action of the NSAIDs have been suggested including a direct effect at the prostaglandin receptor (Collier and Sweatmen, 1968; Rees *et al.* , 1988; Rees and Lopez-Bernal, 1989), and inhibition of histamine and bradykinin release (Lewis and Whittle, 1977; Harris and Vavra, 1985). However, although both peripheral and central mechanisms of NSAID-induced antinociception have been proposed (*vide infra*), it is accepted generally that the mode of action of the NSAIDs, regardless of site, is the inhibition of the formation of the prostaglandins and other endogenous pro-inflammatory mediators (Ferreira *et al.* , 1971; Smith and Willis, 1971; Vane, 1971). Smith and Willis (1971) demonstrated that while prostaglandin production was inhibited in the presence of NSAIDs, the release and activity of PLA_2 was unaffected. Thus, the NSAIDs did not act to prevent the release of arachidonic acid, but instead acted further down the metabolic pathway, but it was not until 1976 that it was proposed that the NSAIDs were acting by inhibiting the action of the enzyme cyclooxygenase (Vane, 1976). The non-steroidal inhibitors of cyclooxygenase may be

classified according to the method by which they inhibit the enzyme: rapid reversible competitive inhibition *e. g.* aryl propionate derivatives such as ibuprofen; irreversible, time-dependent inactivation *e. g.* aspirin, and reversible non-competitive *e. g.* paracetamol (Lands, 1981; Higgs and Vane, 1983). However, it has been suggested that other mechanisms of action must play a role, because NSAIDs which are weak inhibitors of cyclooxygenase *e. g.* carprofen, appear to be as effective clinically as more potent inhibitors of the enzyme (Randall and Baruth, 1976; Strub *et al.* , 1982; Preston *et al.* , 1989). Some of the products of the lipoxygenase pathway have been shown to cause hyperalgesia experimentally (see chapter 3), and a number of NSAIDs including aspirin, diclofenac, indomethacin and ketoprofen, have been reported to inhibit the various lipoxygenase enzymes (Dawson *et al.* , 1982; Paajanen *et al.* , 1982; O'Neill and Lewis, 1989). However, to date, this mechanism of action has not been shown to contribute to the antinociceptive properties of the NSAIDs. Various other actions of the NSAIDs have been proposed to account for the potent anti-inflammatory effects of these drugs, and have been reviewed briefly by Brooks and Day (1991).

1.4.3.2 Antinociceptive actions of the NSAIDs

Salicylic acid was proposed as an analgesic agent by Vulpian in 1880, and towards the end of the 19th century, Dreser suggested the use of aspirin for the same purpose (Lim *et al.* , 1964; Vane and Botting, 1992). For many years it was believed that the analgesic activity of these agents, and other aspirin-like drugs, or non-steroidal anti-inflammatory drugs (NSAIDs), was caused by an effect on the CNS. However, the observations of Randall and Selitto (1957), amongst others (Seigmund *et al.* , 1957; Winder, 1959), suggested that the anti-inflammatory actions of these drugs may have had a peripheral component, and some suggested that the analgesic effects of these agents occurred as a result of the anti-inflammatory actions (Randall and Selitto, 1957). In 1962, Guzman and colleagues showed that the analgesic actions of the NSAIDs did not rely completely on the anti-inflammatory effects of the drugs, by demonstrating that the injection of NSAIDs attenuated the physiological responses indicative of pain after intra-arterial injection of bradykinin in healthy anaesthetised dogs. Moreover, later studies showed that the NSAIDs were effective analgesics at doses which were not anti-inflammatory (Gilfoil *et al.* , 1963; Winter and Flataker, 1965).

Peripheral actions

One of the first major clues to whether the NSAIDs exerted their analgesic effects centrally or peripherally came from the paper published by Lim *et al.* (1964). Using the cross-perfused spleen preparation, the authors observed that bradykinin injected into the vasoisolated, but neurally intact, spleen of a recipient dog caused a physiological pain

response. The response was diminished after administration of morphine systemically to the recipient dog, but not after similar administration to the donor dog. In contrast, injection of NSAIDs in the recipient dog did not reduced the pain response, suggesting that the agents administered did not exert any analgesic effects centrally, whereas injection of the same agents to the donor dog did attenuate the pain response. Consequently, the authors concluded that the NSAIDs exerted their analgesic effects by a peripheral mechanism of action. This proposal was supported by the findings of several other authors subsequently (Guzman *et al.* , 1964; Whittle, 1964; Scott, 1968). It was suggested that the NSAIDs perhaps were acting as antagonists at a peripheral receptor, preventing binding of algesic agents (Lim *et al.* , 1964), however, there also was evidence that the NSAIDs exerted their analgesic actions only in the presence of tissue damage or inflammation (Winder, 1959).

The breakthrough in the investigation of the analgesic activity of the NSAIDs came with 2 important discoveries. In 1969, Willis demonstrated the presence of prostaglandins in inflammatory exudate, and 2 years later Vane (1971) proposed that the NSAIDs acted by inhibiting the formation of the prostaglandins. This suggestion was supported by further work published in the same year (Smith and Willis, 1971; Ferreira *et al.* , 1971). Although Vane's discovery (1971) explained both the anti-inflammatory and anti-pyretic actions of the NSAIDs, it did not appear immediately to provide an explanation for the analgesic effects of this group of drugs.

The prostaglandins had not been shown to be algesic agents, causing instead a hyperalgesia (Horton, 1963; Crunkhorn and Willis, 1969; 1971; Juhlin and Michaelsson, 1969; Willis, 1969; Karim, 1971). The peripheral and central mechanisms of prostaglandin-evoked hyperalgesia are reviewed in chapter 3. Ferreira (1972) demonstrated that aspirin did not attenuate the hyperalgesia caused by subdermal infusion of PGE_1, but suggested that the analgesic action of the NSAIDs could be explained by inhibition of prostaglandin production, thus preventing sensitization of peripheral pain receptors. In the same year, Collier and Schneider (1972) demonstrated that although NSAIDs did not block prostaglandin-induced writhing responses in mice, they did block the writhing responses induced by the intraperitoneal injection of bradykinin and other algesic agents, and they suggested that prostaglandins might be released by such noxious stimuli. In addition, Ferreira *et al.* (1973) showed that bradykinin injected intra-arterially in isolated dog spleen, released prostaglandin-like material, and moreover, they demonstrated that the release of this substance, or substances, was blocked by administration of a NSAID. These findings suggested that the NSAIDs exerted their analgesic actions by preventing the formation of the prostaglandins peripherally, thus inhibiting the sensitization of peripheral nociceptors. These authors also demonstrated that the pseudoaffective responses of increased blood pressure and respiratory rate, evoked after bradykinin was injected into the splenic artery of dogs,

were attenuated by pre-treatment with NSAIDs. Similarly, Moncada *et al.* (1975) demonstrated that the rise in blood pressure recorded after intra-articular injection of bradykinin was attenuated by local injection of NSAIDs, and that the beneficial effects of NSAID injection were lost when exogenous prostaglandin was injected into the joint. Moreover, the authors of the latter study demonstrated that pre-treatment with indomethacin, a NSAID, prevented the increase in locally produced PGE_2 in the dog knee joint induced by carrageenan injection. The findings of the previous studies were reinforced by Ferreira *et al.* (1978a), who demonstrated that local intraplantar injection of NSAIDs attenuated the hyperalgesic response observed after administration of carrageenan. Inhibition of the formation of the prostaglandins in the periphery, thereby preventing sensitization of peripheral nociceptors to the effects of both endogenous and exogenous algesic influences, has remained the most popular explanation of the antinociceptive activity of the NSAIDs (Menasse *et al.* , 1978; Milne and Twomey, 1980; Schweizer and Brom, 1985; Haubrich *et al.* , 1990). However, despite the weight of evidence supporting a peripheral site of antinociceptive action of the NSAIDs, it is possible that antinociception is mediated, at least in part *via* a central mode of action.

Central actions

Towards the end of the last century, a central mode of action for the NSAIDs was claimed by Dreser (*vide supra*), and this suggestion was maintained until challenged by the work of Lim *et al.* (1964) and Guzman *et al.* (1964). In 1978, Ferreira and colleagues (1978a) again proposed a central component to the analgesic effects observed after administration of the NSAIDs. In the latter study the authors observed that both intracerebroventricular (ICV) and intraplantar injection of NSAIDs diminished the hyperalgesia recorded in the rat paw after intraplantar injection of carrageenan. Interestingly, they discovered that previous treatment of one paw with carrageenan reduced the oedema caused by a second injection of carrageenan in the contralateral paw. However, although the intensity of the subsequent hyperalgesia was not reduced, the time to plateau of effect was shorter. These workers also demonstrated that SC-19220, a prostaglandin antagonist, injected either ICV or intraplantar, inhibited the hyperalgesia evoked by injection of carrageenan. Moreover, they showed that ICV injection of prostaglandin enhanced the hyperalgesic response observed after intraplantar injection of prostaglandin. The authors concluded that previous inflammation triggered a central mechanism which accelerated the response to any subsequent inflammatory stimulus, and that this mechanism involved the release of prostaglandins within the CNS. It is interesting to note that later work supported the suggestion that changes within the CNS occur after inflammation, although different mechanisms were proposed (Woolf, 1983; also see chapter 3), and also that spinal administration of prostaglandins has been shown to cause peripheral hyperalgesia (Uda *et al.* , 1990).

Shyu *et al.* (1984) demonstrated that both systemic and central administration of aspirin caused analgesia in monkeys after tooth pulp stimulation, and they showed that to achieve the same level of analgesia a 200 fold increase in dose was required after systemic administration. Moreover, they observed that aspirin-induced analgesia was antagonised by depletion of 5-hydroxytryptamine (5-HT), and enhanced by pre-treatment with either 5-HT or a 5-HT precursor. In a second study, metamizol, a pyrazolone derivative with NSAID activity, increased the response latency to noxious thermal stimulation in rats after both intrathecal and systemic injection, although intrathecal administration in spinal rats was ineffective (Carlsson *et al.* , 1986). The latter workers also demonstrated that metamizol exerted a central analgesic effect involving activation of descending inhibitory systems originating in the periaqueductal grey matter. Intrathecal administration of aspirin and indomethacin in rats also caused analgesia, demonstrated by an increase in latency to response after noxious thermal and mechanical stimulation, although a similar response was not observed after systemic administration of indomethacin (Taiwo and Levine, 1988a), while intrathecal administration of PGE_2 resulted in hyperalgesia to mechanical and thermal stimuli. Moreover, thermal and mechanical analgesia observed after intrathecal administration of morphine was both prevented and reversed by intrathecal injection of PGE_2. Interestingly, the authors demonstrated a synergistic effect between morphine and the NSAIDs administered intrathecally. In the same study, it was shown that PGE_2 administered centrally, blocked analgesia induced by electrical stimulation of the nucleus reticularis paragigantocellularis, while indomethacin reduced the current required to evoke a similar level of analgesia. Furthermore, blocking or destroying spinal noradrenergic synapses prevented the development of the effects observed after spinal administration of PGE_2 or NSAIDs. More recently, Bjorkman *et al.* (1990) demonstrated that diclofenac exerted a central, naloxone-reversible antinociceptive action in rat writhing and colo-rectal distension models after both central and systemic administration. It is interesting to note that injection of diclofenac had previously been shown to be linked to an increase in hypothalamic, and a decrease in pituitary β-endorphin, in association with increased circulating levels of the opioid peptide (Sacerdote *et al.* , 1985). Moreover, it was demonstrated recently that administration of flunixin meglumine to sheep caused a naloxone-reversible analgesia, and the authors proposed the involvment of a descending opioidergic system in NSAID-induced analgesia (Chambers *et al.* , 1993b).

Electrophysiological studies also have suggested a central component to NSAID-induced analgesia (Guilbaud *et al.* , 1982; Yezierski *et al.* , 1983; Carlsson *et al.* , 1988; Groppetti *et al.* , 1988; Jurna and Brune, 1990; Jurna *et al.* , 1992). Guilbaud *et al.* (1982) investigated the activity of ventrobasal thalamic neurones in rats with experimentally-induced arthritis, and showed that systemically administered aspirin depressed neuronal responses to joint

manipulation. Further studies have investigated the effects of the NSAIDs on evoked activity of thalamic neurones in the normal animal, and shown that these agents dose-dependently inhibit evoked responses in a naloxone irreversible manner, and that unlike morphine the NSAIDs do not completely block evoked activity (Carlsson *et al.* , 1988; Groppetti *et al.* , 1988; Jurna and Brune, 1990; Jurna *et al.* , 1992). In addition, Groppetti *et al.* (1988) reported that lysine acetylsalicylate decreased the concentration of met-enkephalin immuno-reactive derivatives in several areas of the brain, although concentrations of 5-hydroxyindole acetic acid, a metabolite of 5-HT, increased. Moreover, pre-treatment with a 5-HT receptor antagonist attenuated the antinociceptive activity of the NSAID, and administration of both tryptophan or 5-hydroxytryptophan mimicked the effects of the NSAID *i. e.* increased turnover of 5-HT and reduced evoked neuronal activity.

Several central sites of action of the NSAIDs have been proposed: preoptic anterior hypothalamus (Shyu *et al.* , 1984; Shyu and Lin, 1985); lateral hypothalamus (Dubas and Parker, 1971); periaqueductal grey matter (Carlsson *et al.* , 1986) and the thalamus (Carlsson *et al.* , 1988). Moreover, several mechanisms for the central analgesic action of the NSAIDs have been suggested (*vide supra*) (Shyu *et al.* , 1984; Shyu and Lin, 1985; Groppetti *et al.* , 1988; Taiwo and Levine, 1988a). Recently, Malmberg and Yaksh (1992a) demonstrated that spinal administration of NSAIDs attenuated the hyperalgesic response observed after spinal injection of both substance P and N-methyl-D-aspartate agonists, and pre- and post-treatment with the NSAIDs were shown to be equally effective at limiting hyperalgesia. In a second study, the same authors investigated the central effects of various NSAIDs from several different chemical groups including, acetylsalicylic acid, ketorolac, indomethacin, acetaminophen, zomepirac, flurbiprofen and ibuprofen (racemate and S(+)-isomer and R(-)-isomer) (Malmberg and Yaksh, 1992b). This was an important investigation because, although a large number of studies had been conducted into the central mechanisms of NSAID-induced antinociception, only a limited number of agents had been investigated, primarily indomethacin and aspirin. The authors of the latter study described a dose-dependent, stereospecific, submaximal suppression of the second phase of the response to injection of formalin into the rat paw, after both spinal and systemic administration of the NSAIDs previously mentioned. It is of interest that the drugs were 100 to 1000 times more potent after spinal injection, were effective both pre- and post-formalin injection and that when administered intrathecally, they had little effect on the escape response generated by the hot-plate test.

Experimental evidence supporting a central site of antinociceptive action of the NSAIDs is accumulating, but presently, only a limited number of clinical studies have been reported. Intrathecal administration of lysine acetylsalicylic acid has been shown to provide relief from pain in patients with cancer (Devoghel, 1983), and more recently, Willer *et al.* (1989), using

the threshold of the nociceptive flexion reflex from the biceps femoris muscle, elicited by electrical stimulation of the sural nerve, demonstrated a supraspinal mode of action for ketoprofen. Obviously, there is a need for further clinical studies to be conducted, not only in humans, but also in the domestic species.

1.4.3.3 Other actions of the NSAIDs

In addition to their antinociceptive effects, the NSAIDs exert 2 other main pharmacological actions, anti-inflammatory effects and anti-pyretic effects. While the anti-inflammatory actions of the NSAIDs are very important, and these drugs are prescribed widely for the control of many inflammatory conditions, including both acute and chronic inflammatory musculoskeletal conditions, only a brief review of the anti-inflammatory effects will be given. Many of the products of arachidonic acid metabolism are important mediators of the inflammatory response, characterised by the signs of swelling, erythema, heat, pain and loss of function (Higgins and Lees, 1984). These signs may be explained in part by the action of the eicosanoids, in combination with other chemical and cellular responses *e. g.* many of the prostaglandins, and some of the products of the lipoxygenase pathways mediate hyperalgesia in association with other endogenous chemical mediators (see chapter 3); PGE_2 and PGI_2 mediate vasodilation; the hydroxyeicosatetraenoic acids are potent chemotaxins; LTB_4 is a potent chemotaxin for polymorphonuclear leukocytes and affects vascular permeability and TXB_2 promotes platelet aggregation and vasoconstriction (Higgins and Lees, 1984; Lees and Higgins 1985; Lees *et al.* , 1991a). In the treatment of inflammatory conditions, removal, or treatment directed at the initiating factor, is of primary importance, however drugs which directly or indirectly modulate the activity of the chemical mediators of inflammation, such as the NSAIDs, also play an important role in limiting the effects of inflammation. It is interesting to note that the dose of many NSAIDs required for antinociception is frequently less than that required for anti-inflammatory effect (Dawson and Willoughby, 1985).

A comprehensive review of the role of the eicosanoids in pyresis is given by Milton (1992). Briefly, pyresis occurs as part of the host acute phase response in response to infection, inflammation and tissue damage, and is mediated by interleukin-1 which activates PLA_2, thus stimulating arachidonic acid metabolism and increasing production of prostaglandins (see Dinarello, 1984). Milton and Wendlandt (1970) demonstrated that intracerebroventricular injection of a variety of prostaglandins caused symptoms similar to those observed after injection of bacterial endotoxin, but which in contrast, were not controlled by administration of paracetamol. Consequently, the authors proposed that PGE_1 acted as a modulator of temperature regulation. Since the latter report, the area has been researched extensively, and the role of prostaglandins in the induction and maintenance of

fever is well established (see Milton, 1992). The Reverend Stone (1763) described the use of an extract of willow bark in the treatment of fever, and since that account, salicylates and other NSAIDs have been used to control pyresis, acting to prevent the production of prostaglandins by inhibiting fatty acid cyclooxygenase.

The prostaglandins and leukotrienes, especially LTB_4, may act to contract or relax smooth muscle in the vascular system (Bergstrom *et al.* , 1968; Piper, 1984; Bhagwat *et al.* , 1985), but in addition, the eicosanoids can cause similar effects on bronchial and tracheal smooth muscle (Spannhake *et al.* , 1981; Piper, 1984), uterine smooth muscle (Bergstrom *et al.* , 1968) and gastrointestinal smooth muscle (Wilson and Kaymakcalan, 1981). Moreover, the eicosanoids influence gastrointestinal secretion (Wilson and Kaymakcalan, 1981), and play an important regulatory role in the kidney (Dunn and Zambraski, 1980). Finally, TXA_2, a major product of arachidonic acid metabolism in platelets (Hamberg *et al.* , 1975), causes platelet aggregation, and the NSAIDs, in particular, aspirin, may be used for the treatment and prevention of thrombotic conditions, because of the anti-aggregatory effects exerted by these drugs as a consequence of cyclooxygenase inhibition.

1.4.3.4 Adverse effects of NSAIDs

In 1986, one quarter of all adverse drug reactions reported to the Committee on Safety of Medicines in the United Kingdom were due to administration of NSAIDs. Administration of these drugs can be associated with a wide range of side effects including renal toxicity, gastrointestinal toxicity, hepatotoxicity, prolongation of pregnancy and teratogenicity, increased bleeding time, bone marrow depression and blood dyscrasias, skin reactions, central nervous system disturbances and hypersensitivity reactions, all of which are related to inhibition of eicosanoid production (Rainsford, 1984a; 1984b). The renal and gastrointestinal adverse reactions perhaps are 2 of the most serious side effects encountered. Prostaglandins are important in the autoregulation of renal blood flow and glomerular filtration, and influence tubular transport of ions and water (Dunn and Zambraski, 1980). Administration of NSAIDs has been associated with a variety of renal problems in both humans and animals, including acute renal failure, papillary necrosis, nephrotic syndrome and interstitial nephritis (see Rubin, 1986). The role of the prostaglandins in the gastrointestinal tract has been reviewed and includes, inhibition of acid secretion in the stomach, inhibition of gastrin and pancreatic secretion, stimulation of salivary and biliary secretions, modulation of gastrointestinal blood flow and motility and finally, cytoprotection (Highland and Upson, 1986). Therefore, the prostaglandins play an important role in the normal physiological processes occurring within the gastrointestinal tract, and administration of NSAIDs can induce a variety of effects, including gastric erosion, peptic ulcer formation and perforation and changes in the permeability of the bowel (Brooks and Day, 1991).

1.5 Footrot in sheep

Footrot is a contagious disease of ruminants which is transmitted by *Dichelobacter nodosus* (previously referred to as *Bacteroides nodosus*), and sheep are very susceptible to the disease (Stewart, 1989). Footrot has been recognized for more than 250 years in all countries where sheep are reared commercially (Deane and Jenson, 1955), and in addition to the important welfare implications, it also can cause large economic losses through decreased productivity and labour intensive treatment (Stewart, 1989).

There are 2 main forms of footrot which occur clinically, virulent, or progressive, and benign, or non-progressive, and it has been suggested that the severity of the disease depends on the invasiveness of the *Dichelobacter spp.* (Stewart *et al.* , 1986). Lameness is the most prominent clinical sign associated with both forms of the disease (Morgan, 1987; Stewart, 1989), and Stewart (1989) describes the pathological and clinical findings changes associated with the different forms of the disease. Briefly, virulent footrot is a persistent condition, characterised by presence of a necrotic, foul smelling exudate with extensive under-running and separation of the horn of the hoof wall and sole in a large number of sheep within the flock. However, benign footrot is limited generally to inflammation of the interdigital skin, with very little separation of the hoof and little exudation, but again it may be present in a large number of sheep within the flock.

Footrot has a seasonal incidence, occurring in warm moist conditions especially when pasture is lush underfoot (Beveridge, 1938), and although a wide variety of organisms can be isolated from infected feet, the 2 organisms found most commonly are *Dichelobacter nodosus* (Beveridge, 1941) and *Fusobacterium necrophorum* (Mohler and Washburn, 1904). British breeds of sheep, and their crosses, have a greater natural resistance to footrot than do the Australian Merino (Emery *et al.* , 1984), and it has been shown that susceptibility to footrot is a heritable characteristic and that natural resistance to footrot may be increased by selective breeding (Stewart, 1989). Fortunately, in addition to paring feet, there are a variety of treatment options for footrot, including both topical and parenteral therapies, and vaccination against infection with *Dichelobacter ssp.* is possible. Foot care in sheep, and treatment options against footrot have been reviewed by Boundy (1979), Morgan (1987) and Stewart (1989).

Studies described in this thesis were undertaken to examine the antinociceptive effects of the non-steroidal anti-inflammatory drugs in healthy sheep and also in sheep suffering from chronic and acute pain. The initial aim of these studies was to develop a test system in sheep whereby the antinociceptive properties of the NSAIDs could be detected, and subsequently, to evaluate the efficacy and potency of these drugs as antinociceptive agents in this species.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Animals

A group of 8 experimental sheep (Suffolk cross and Border Leicester x Scottish Blackface (Greyface)), aged 1 to 2 years, were purchased from either the University farm or from Mr. J. Struthers, High Currochtrie Farm, Drummore, U. K. Clinical examination indicated that the sheep were healthy and had normal foot conformation. Moreover, the sheep had no conformational changes of the feet suggestive of previous bacterial infection. The sheep were maintained indoors as a group and fed on hay (*ad libitum*) and concentrates. Water was available *ad libitum*.

The sheep were treated with a total spectrum wormer, albendazole (Valbazen, 2.5 %, Smithkline Beecham Animal Health, Tadworth, U. K.) every 6 months and were vaccinated yearly against *Clostridium ssp.* (Heptavac-P, Hoechst Animal Health, Milton Keynes, U. K.). Foot inspection was carried out on a fortnightly basis, and foot trimming was carried out as required. The sheep were weighed prior to all experiments (weights ranged between 45 and 99 kg).

The remaining experimental sheep (study 1 and study 2, chapter 3) were owned by the University farm and were all Greyfaces. These sheep were maintained at grass from April to December. For the remaining time they were maintained indoors in groups of up to 30 sheep and were fed hay (*ad libitum)* and concentrates. Water was available *ad libitum*. The sheep were weighed prior to all experiments (weights ranged between 45 and 66 kg).

Sheep used for studies of subjective assessment of lameness were owned by Mr. J. Blair, Capellie Farm, Barrhead, U. K. (Suffolk cross) (study 1, chapter 7) and Mr. I. Lamont, Tandalmuir Farm, Lochwinnoch, U. K. (Scottish Blackface) (study 2, chapter 7).

Sheep used in the study investigating changes in thresholds to noxious stimulation post operatively (study 3, chapter 3), were owned by the Institute of Animal Physiology and Genetics Research, Roslin, U. K. In addition, a further 4 sheep were purchased from Mr J. Struthers. All sheep were Scottish Blackface ewes aged 3 to 4 years and were maintained outdoors. Water was available *ad libitum*. Twelve hours before the experiment the sheep were weighed (range 43 to 64 kg), brought indoors, maintained on wood shavings and fasted prior to the induction of general anaesthesia. The sheep were returned to the field 24 hours after surgery or anaesthesia.

2.2 Drugs and drug administration

The following drugs were used:

Buprenorphine- buprenorphine hydrochloride (Temgesic, 300 µg/ml, Reckitt and Colman Pharmaceuticals, Hull, U. K.).

Carprofen- (50 mg/ml, donated by Grampian Pharmaceuticals Ltd., Glasgow, U. K.).

Fentanyl- fentanyl citrate (Sublimaze, 50 µg/ml, Janssen Pharmaceutical Ltd. Oxford, U. K.).

Flunixin - flunixin meglumine (Finadyne, 50 mg/ml, Schering-Plough Animal Health, Mildenhall, U. K.).

Halothane- (Fluothane, Coopers Pitman-Moore, Crewe, U. K.).

Lignocaine- lignocaine hydrochloride (Lignavet, 20 mg/ml, C-Vet Ltd., Bury St Edmunds, U. K.).

Penicillin- procaine penicillin (150 000 iu) and benzathine penicillin (150 000 iu) (Duphamox LA, Duphar Veterinary Ltd., Southhampton, U. K.).

Sterile water- (Water for Injections, RMB Animal Health Ltd., Dagenham, U. K.).

Thiopentone- thiopentone sodium (Intraval Sodium, RMB Animal Health Ltd.).

Xylazine- xylazine hydrochloride (Rompun, 20 mg/ml, Bayer UK Ltd., Bury St Edmunds, U. K.).

Ketamine- ketamine hydrochloride (Vetalar, 100 mg/ml, Parke-Davis Veterinary, Pontypool, U. K.)

Drugs were diluted, if necessary, in sterile 0.9 % saline (Aquapharm 0.9 % w/v sodium chloride intravenous infusion BP, Animalcare Ltd., York, U. K.). Thiopentone sodium (5 %) was made up from the dried substance by dissolving in water for injection. All drugs were administered by intravenous injection (IV), unless otherwise stated, into a jugular or cephalic vein *via* a sterile needle (Microlance, 25 mm long, 0.9 mm or 0.5 mm diameter, Becton-Dickenson, Dublin, Ireland).

2.3 Chemical Materials

All water used in the preparation of standard solutions was distilled in the presence of potassium permanganate. 'Analar' or 'Aristar' grade chemicals (BDH Chemicals Ltd. , Poole, U. K.) were used in the preparation of all solutions.

Solutions of sodium hydroxide (Koch-Light Ltd. , Haverhill, U. K.), di-sodium hydrogen orthophosphate (BDH Chemicals Ltd.) and citric acid (Sigma Chemical Company Ltd. , Poole, U. K.) were prepared in water.

Citrate phosphate buffer (pH 3)

Stock citrate phosphate buffer solution (pH 3) was made from 20.5 ml of di-sodium hydrogen orthophosphate solution (0.2 M) and 79.5 ml of citric acid (0.1 M). The final pH of the solution was adjusted by the addition of sodium hydroxide solution (1 M) or orthophosphoric acid .

Citrate phosphate buffer (pH 5)

Stock citrate phosphate buffer solution (pH 5) was made from 49.3 ml of di-sodium hydrogen orthophosphate solution (0.2 M) and 50.7 ml of citric acid (0.1 M). The final pH of the solution was adjusted by the addition of sodium hydroxide solution (1 M) or orthophosphoric acid.

The pH of the buffer solutions was checked using a digital pH / temperature meter (Fisons Scientific Equipment).

Flunixin meglumine and carprofen reference standards were donated by Schering-Plough Animal Health and Grampian Pharmaceuticals Ltd. , respectively.

2.4 Blood sampling techniques and sample preparation

Blood for drug analysis was collected *via* an intravenous cannula (Vygon Intraflon 2, 80 mm long, 2.1 mm diameter, Vet Drug Company plc, Falkirk, U. K.) from a jugular vein. Prior to cannulation, wool overlying the jugular furrow was clipped and the area cleaned. Local anaesthesia was achieved by the intradermal and subcutaneous injection of 1 ml of lignocaine hydrochloride into the area where the cannula was to be inserted. A small skin incision was made with a sterile scalpel blade, and the cannula inserted through the skin incision into the jugular vein. The cannula was sutured in position, a three-way tap (Vet Drug Company plc) attached and the cannula flushed with 3 to 5 ml of heparinized saline (4 units per ml, 0.25 ml heparin sodium injection BP (mucous), 1000 units per ml, Evans, Langhurst, U. K. diluted

in 500 ml sterile saline (Animalcare Ltd.)). Subsequently, the cannula was flushed with the same solution as required.

Before blood was collected for analysis, approximately 3 ml of blood was discarded to remove any remaining heparinised saline from the cannula. Ten ml blood samples were collected into lithium heparin syringes (Li-Heparin LH/10 Monovette®, Sarstedt Ltd. , Loughborough, U. K.). Following centrifugation at 1500 *g* for 10 minutes in a cooled (5-15°C) centrifuge (MSE Chilspin, M. S. E. Scientific Instruments, Crawley, U. K.), plasma was decanted into 10 ml neutral tubes (Sarstedt Ltd. , Loughborough, U. K.), labelled, and stored at -20°C until analysed by high performance liquid chromatography (HPLC) for either flunixin or carprofen racemate.

2.5 Preparation of carrageenan (0.0625 %)

A solution of the sodium salt of lambda-rich carrageenan (0.0625 %) (Viscarin, F. M. C. Corporation, Springfield, U. S. A.) was prepared by dissolving 0.00625 g of carrageenan in 10 ml of 0.9 % saline. The solution was placed in a sonic bath (Sonicleaner, Dawe Instruments Ltd. , Coatbridge, U. K.) to aid dissolution of the carrageenan. Aliquots (2 ml) of the solution were placed into 5 ml glass tubes, the tubes capped with rubber stoppers and a needle (Microlance, 25 mm long, 0.9 mm diameter, Becton-Dickenson) placed through the rubber stopper. The tubes were placed in a metal rack and sealed in an autoclave bag (Autoclavable Disposal Bag, Guest Medical, Edenbridge, U. K.) prior to autoclaving (Sterilin Instruments, Model Number 704-8000-DSE, T. I. S. Services, Alton, U. K.) to sterilise the solution. Before removing the tubes from the bag at the end of the autoclave cycle, the needles were removed from the rubber stoppers to maintain sterility of the carrageenan solution once the bag was opened. The solution was stored for a period of 2 weeks (but no longer than 3 weeks) prior to the experiments.

2.6. Intradermal injection

The wool, or hair, overlying the site of injection was clipped 24 hours before the experiment. Intradermal (ID) injection was made using a sterile needle (Microlance, 10 mm long, 0.45 mm diameter, Becton-Dickenson) inserted into the skin parallel to the surface. If the needle became dislodged, the site began to bleed, or the solution which was injected was seen to ooze from the site, a second injection was made at least 2.5 cm from the first.

2.7 Preparation of zinc sulphate/sodium lauryl sulphate solution (10 %)

A solution of zinc sulphate (10 %) (Golden Hoof, Shep Fair Products, Crickhowell, U. K.) was prepared by dissolving 3 kg of compound in 5 l of warm tap water. The solution was placed into a washed sheep footbath and diluted to a final volume of 30 l.

2.8 Tourniquet

A pneumatic tourniquet was constructed according to the method described by Ibrahim (1989). Briefly, a length of rubber tubing, approximately 35 cm, was cut from the inner tube of a bicycle tyre, leaving the valve attached. The ends of the tubing were clamped using thin metal clamps, to ensure an airtight seal. The inner tube was sewn into a tightly fitting fabric sleeve and self-adhesive fabric was stitched in place to hold the tourniquet around the limb. A bicycle pump connector was attached to the valve. The distal end of the connector was cut off and attached to a three way tap. The inflated tourniquet was tested for leaks by inflating it to a pressure >300 mm Hg and submerging it in water.

The tourniquet was applied to the forelimb of the sheep proximal to the carpus and distal to the elbow, and was inflated using a sphygmomanometer (Thackray Ltd. , Glasgow, U. K.) to a pressure of 300 mm Hg. A photograph of the tourniquet is shown in Fig. 2.1.

2.9 Mechanical threshold testing unit

A device for testing thresholds to noxious mechanical stimulation (Chambers *et al.* , 1990) was purchased from the Department of Pharmacology, University of Bristol, Bristol, U. K. (Fig. 2.2) . Briefly, a unit was applied to the leg of the sheep just proximal to the carpus and secured by a length of self adhesive fabric which ran through a fulcrum, thus allowing the unit to be positioned securely. The leg unit consisted of a pneumatically driven 2 mm diameter blunt-ended pin which applied a mechanical noxious stimulus to the leg. The pin was attached to a baseplate which acted as a loose piston and which was enclosed fully in a small circular box. Above the piston a rubber balloon was positioned and secured to the hub section of a 5 ml syringe, which in turn was connected to the 'operate' port on the control unit (Fig. 2.3) by a length of polyvinylchloride tubing (internal diameter 3 mm, Portex Ltd. , Hythe, U. K.). The device was driven by compressed oxygen from a size D cylinder (BOC Medical Gases, Guildford, U. K.). A reducing valve for oxygen (BOC Medical Gases) was attached to the cylinder, and the oxygen passed through a micro-adjustable needle valve. The adjustable valve allowed the rate of increase of force applied to the leg to be varied. The rate of increase of force was adjusted to achieve a response within 20 to 30 seconds in normal

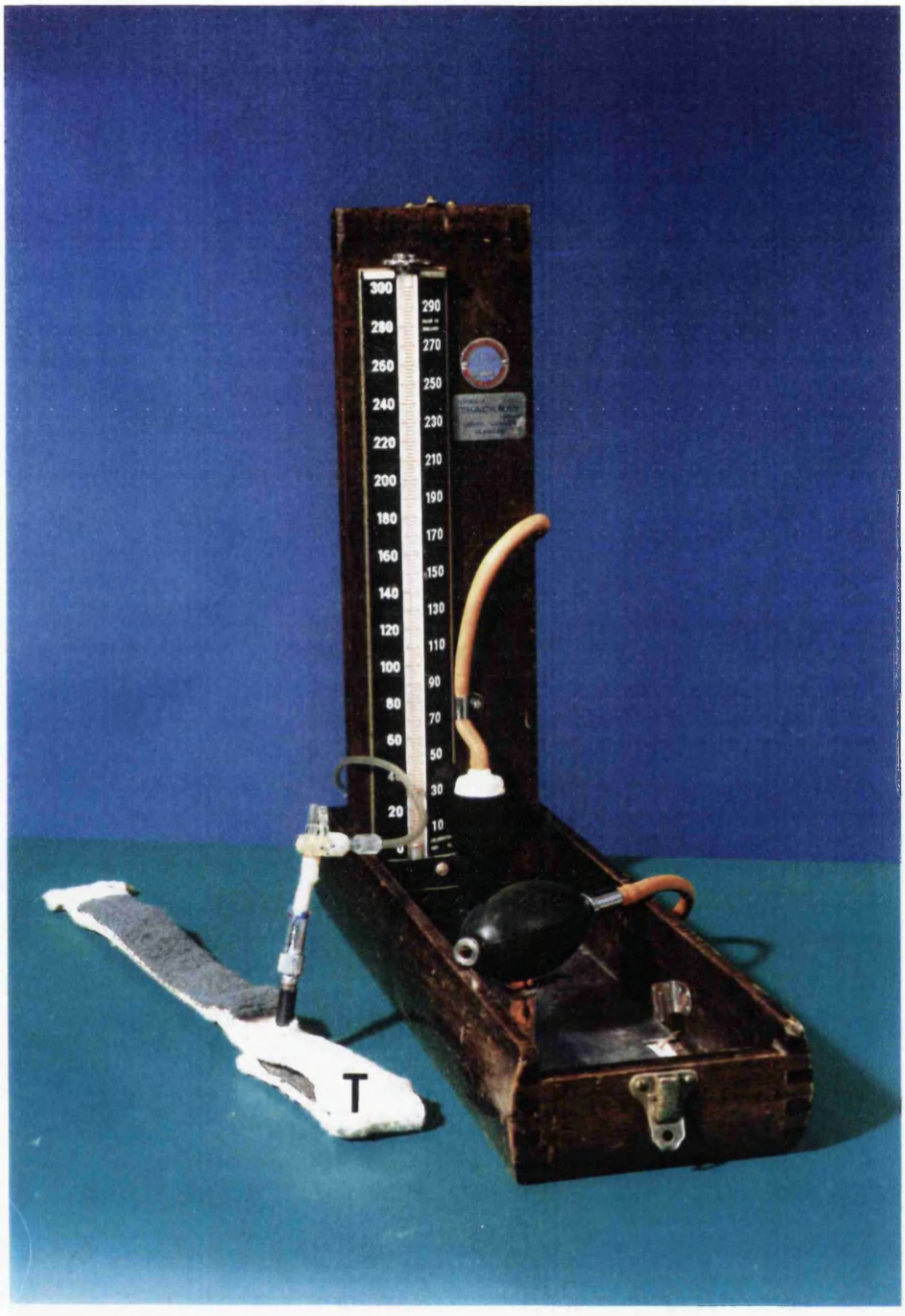

Figure 2.1. Photograph of a pneumatic tourniquet (**T**) and the sphygmomanometer used to inflate the tourniquet.

The tourniquet is inflated to a pressure of 300 mm Hg.

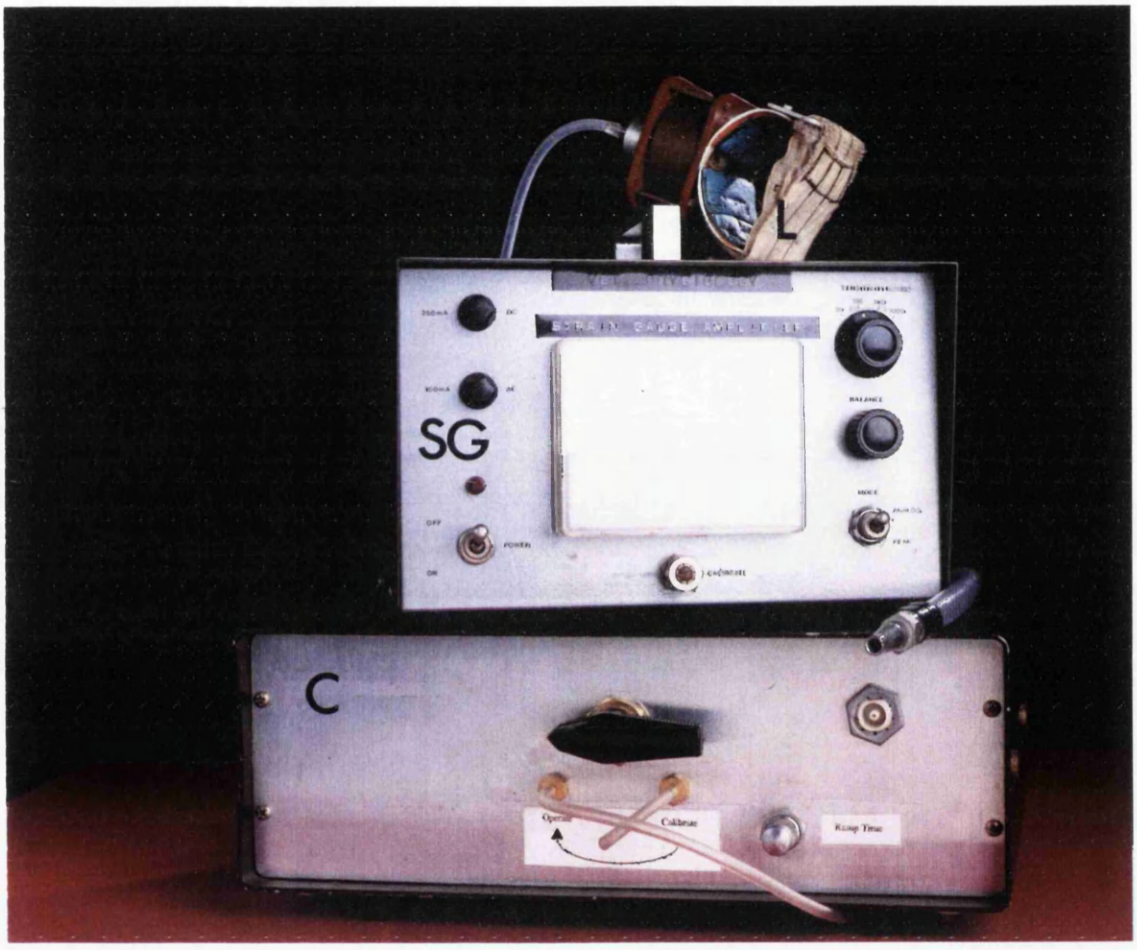

Figure 2.2. Photograph of apparatus for the production of mechanical nociceptive stimuli.

L leg unit

S G calibrated strain gauge amplifier

C control unit

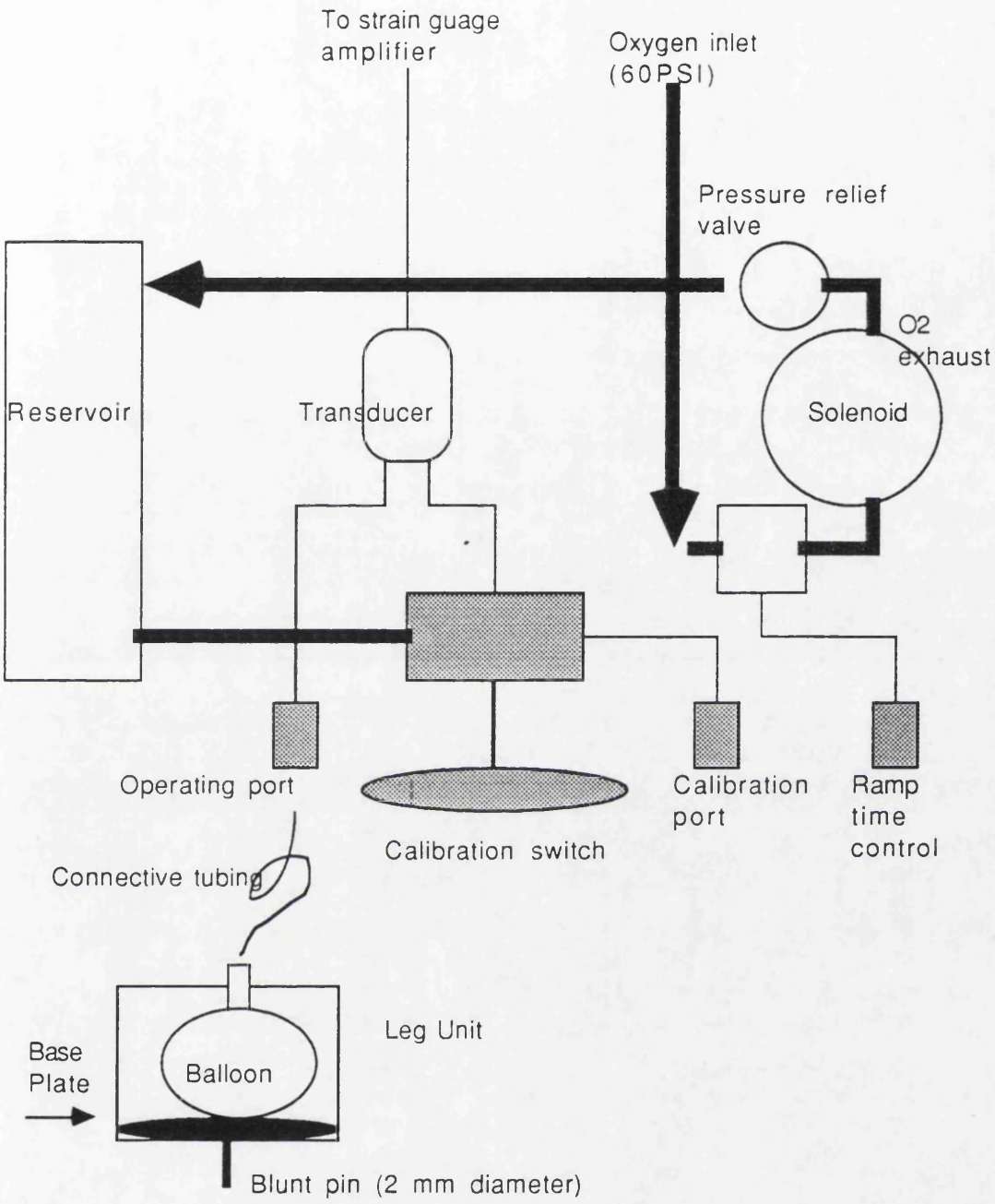

Figure 2.3. Schematic representation of apparatus for the production of mechanical nociceptive stimuli.

sheep. A solenoid-operated three way valve diverted the compressed gas either *via* a reservoir and pressure transducer to inflate the balloon, or *via* an exhaust valve, thus removing the stimulus from the leg. The reservoir was connected to a pressure relief valve which opened when the pressure within the system rose to 20.7 kPa. A schematic diagram of the apparatus for the production of mechanical nociceptive stimuli is shown in figure 2.3.

The pressure transducer was connected to a strain gauge amplifier which was calibrated in mm Hg with a mercury manometer (Portable manometer P200, Digitron Instrumentation, Hertford, U. K.). Calibration was checked weekly.

2.10 Thermal threshold testing unit

A thermal threshold testing unit, similar to the device described by Nolan *et al.* (1987a), was constructed (Chemistry Workshop, University of Glasgow). Briefly, the unit comprised of an ear clip, a junction box and a control unit (Fig. 2.4). The ear clip was constructed from a metal bulldog clip and housed an 8 ohm resistor, as the heating element, covered by a small strip of copper to ensure rapid and even heat distribution. A thermocouple, the tip of which was located between the copper strip and resistor, was used to detect temperature changes in the heating element. The ear clip was attached to the main control unit *via* a junction box. The following modifications were incorporated into the original design for this control unit. To improve memory accuracy, a high performance operational amplifier (Bifet™ Op-Amps, RS Components, Corby, U. K.) was used to replace the original operational amplifier, which removed the need for time consuming off-set nulling techniques. A diode was added to the memory board between the input from the manually-operated stop button and the ramp reset relay. This facilitated the rapid cooling of the ear clip heating unit when the device was in stop mode. A general purpose small signal transistor (NPN, BC108 transistor, RS Components) was used to replace the stop mode stabilisation resistor, speeding up data acquisition without encountering significant memory creep. Finally, a voltage divider was used for the heater ramp control and the over temperature control *i. e.* the cut-off control, allowing a larger range of adjustment with finer control.

The main features of the control unit are shown in Fig. 2.4. The unit was operated by pressing a 'run' button, and could be stopped manually . A remote control (ext. control) also could be attached to the main control unit and used to switch it on and off. A digital readout of temperature was given, and could be used either as a transient read-out of current temperature, or in conjunction with a 'peak hold' facility, which recorded the peak temperature reached at the resistor in the ear unit at cut-off. A 'reset' button was used in conjunction with the peak hold facility, returning the digital temperature readout to a

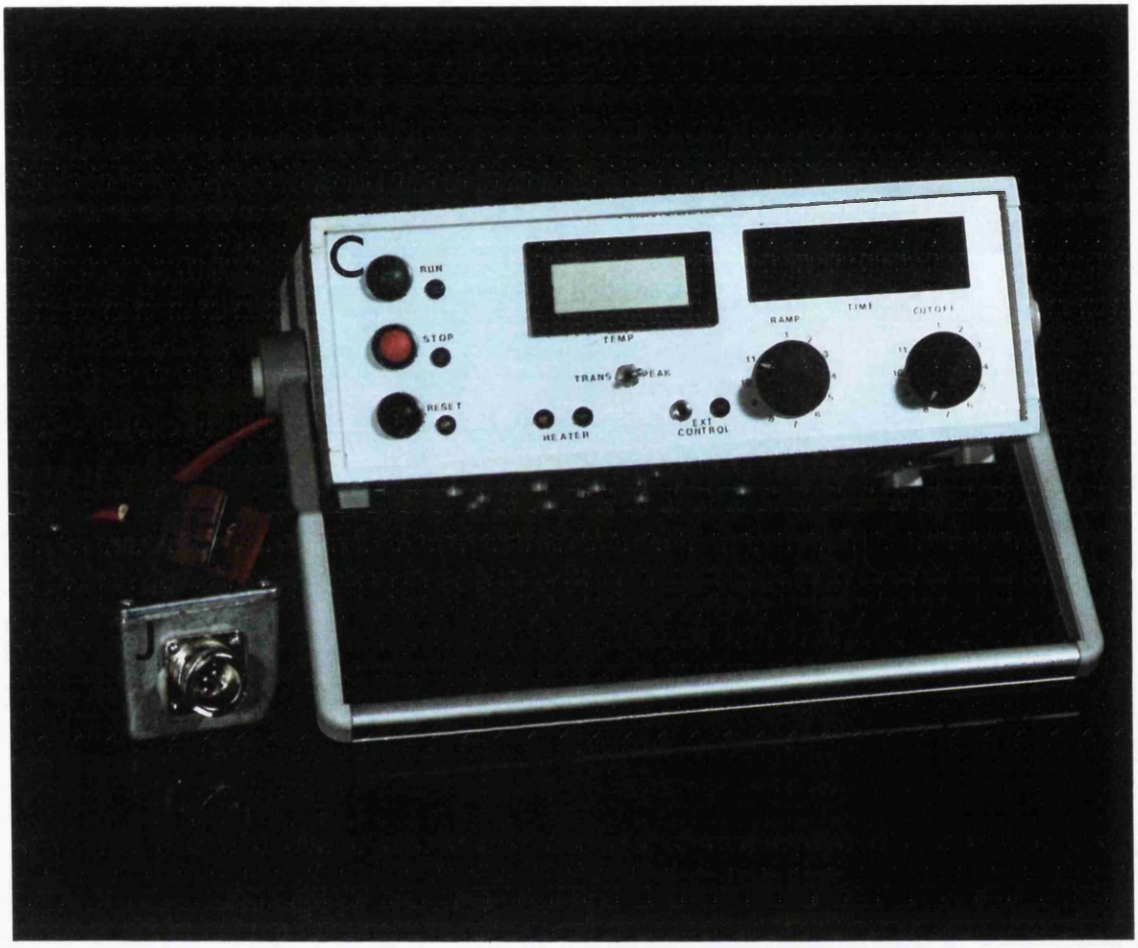

Figure 2.4. Photograph of apparatus for the production of thermal nociceptive stimuli.

C control unit

E ear clip

J junction box

preset temperature, which is adjustable from within the unit, and eliminates unnecessary inaccuracies in the run time. This feature also was included in the original design. An adjustable ramp, and variable cut-off temperature were features of the unit.

Calibration of the thermal unit was achieved using a thermocouple to connect the copper tubing encasing the resistor to a multimeter (Multimeter 4055). The unit, which incorporated a facility to break the circuit to the ear clip in the event of thermocouple damage, was calibrated monthly, or more regularly as required.

2.11 Statistical analysis

All statistical analyses were carried out using Minitab Release 8 (Macintosh® version, Clecom, Birmingham, U. K.).

CHAPTER 3

THE ANTINOCICEPTIVE EFFECTS OF NSAIDS IN SHEEP:

1 CLINICAL PAIN

3.1 Introduction

It is agreed that pain is a subjective phenomenon, and as such it is difficult to provide a universally acceptable definition. In 1986, the International Association for the Study of Pain defined pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage (Merskey, 1986). Woolf (1989; 1991) divided pain into 2 separate and qualitatively different categories, physiological and pathological pain. Physiological pain was described as a localised, normal, brief, protective response to a noxious stimulus in a normal animal, which resulted in either no, or only localized inflammatory tissue damage. In contrast, pathological pain resulted in the following phenomena: pain may be present in the absence of a noxious stimulus (persistent pain); responses to noxious stimuli may result in an exaggerated response (hyperalgesia); thresholds to noxious stimuli may be lower than in normal animals (allodynia); pain may be perceived at a site distant to the original site of injury (referred pain) and interactions between the sympathetic and somatosensory systems may occur. The development of allodynia and hyperalgesia are the common denominators of pathological pain of all origins. The classical example of hyperalgesia frequently quoted in review articles (Higgs and Moncada, 1983) is that of sunburned skin. The affected skin may not be painful until touched or immersed in warm water, when exaggerated responses occur. Indeed, Hardy *et al.* (1950) demonstrated that the threshold to thermal stimulation in human skin was reduced by as much as 50 % after ultra violet irradiation of the skin.

Previously, hyperalgesia had been categorised into primary and secondary hyperalgesia (Lewis, 1935; Hardy *et al.* , 1950). Primary hyperalgesia, is a reduction in the threshold to noxious stimulation in the area local to the peripheral site of injury, while secondary hyperalgesia is a reduction in the threshold to noxious stimulation in surrounding uninjured tissue (Thalhammer and LaMotte, 1982; Raja *et al.* , 1988). Primary hyperalgesia results in an increased sensitivity to both thermal and mechanical stimulation while secondary hyperalgesia generally results in increased sensitivity to mechanical stimulation only (Campbell *et al.* , 1979; Raja *et al.* , 1984), although increased sensitivity to thermal stimulation has been recorded (Fitzgerald, 1979). Primary hyperalgesia is assumed to result from changes in the sensitivity of nociceptors in the area immediately adjacent to the injury, with sensitization of both C- and A-fibre nociceptors having been observed (Raja *et al.* , 1988). Secondary hyperalgesia may occur as a result of the antidromic spread of action potentials to adjacent peripheral branches of the same nerve with local release of hyperalgesic mediators (Lewis, 1935; LaMotte *et al.* , 1984), or it may involve central mechanisms (Hardy *et al*, 1950; Fitzgerald, 1979).

3.1.1 Peripheral sensitization

Sensitization of peripheral nociceptors adjacent to the site of injury has been recorded (Lynn and Carpenter, 1982; Fleischer *et al.* , 1983), however, this was not a constant feature of all units tested and similarly, an increase in spontaneous activity was not observed in every case. Fitzgerald (1979) demonstrated that a small skin incision made outside the receptive field of an isolated polymodal nociceptor unit with unmyelinated axons, induced the development of spontaneous activity and lowered thresholds to thermal stimulation within the receptive field of the unit under investigation. This phenomenon is regarded as secondary hyperalgesia. Primary hyperalgesia *i. e.* reduced thresholds to thermal stimulation, also was observed on repeated heating within the receptive field of the isolated nociceptor unit. Moreover, Fitzgerald (1979) demonstrated that antidromic stimulation of a nerve proximal to the site of stimulation also induced the development of sensitization. Although, blockade at the site of injury by local anaesthetics prevented the development of secondary hyperalgesia, it did not abolish totally the evoked spontaneous activity. Consequently, it was suggested that evoked spontaneous activity and the mechanisms by which sensitization occur may be different. The peripheral neural mechanisms of primary and secondary hyperalgesia have been reviewed (Raja *et al.* , 1988).

Woolf (1983) demonstrated that pain hypersensitivity following a localized thermal injury in decerebrate rats with intact flexor withdrawal reflexes, was shown by alterations in both the threshold to stimuli and the responsiveness of the flexion reflex. Electrophysiological studies in these rats showed that in ipsilateral flexor efferents, spontaneous activity increased while the Von Frey hair threshold fell in both the ipsi and contralateral mechanoreceptive fields. The changes evident with electrophysiological studies shadowed the changes observed physically, where a bilateral increase in both the response and duration of response to a standard pinch (150 g/mm^2) was observed. Woolf (1983) commented that nociceptor sensitization could account for the ipsilateral changes observed, but that this explanation could not account for the hyperalgesia and allodynia seen in association with an expansion of receptive fields on the contralateral side. He concluded that pain hypersensitivity following thermal injury was related to changes within the central nervous system as well as changes at the site of injury, a theory supported by the findings of Coderre and Melzack (1987), who demonstrated that spinal anaesthesia, induced immediately following a peripheral thermal injury, limited the development of contralateral hyperalgesia.

3.1.2 Central sensitization

Central sensitization may be regarded as facilitated central responses occurring in response to normal afferent input. It has been shown that brief C-fibre input into the spinal cord results in heterosynaptic facilitation and a prolonged increase in the excitability of the flexion reflex in decerebrate rats, with muscle C-fibre afferents evoking longer lasting changes than cutaneous C-fibre afferents (Wall and Woolf, 1984; Woolf and Wall, 1986a). However, facilitation was not shown to occur when myelinated A-fibres were activated in a similar manner (Wall and Woolf, 1984). Such changes have been reported following experimentally-induced unilateral inflammation in rats (Hylden *et al.* , 1989). Electrophysiological recordings from dorsal horn neurones revealed an increase in receptive field, and in ongoing or bursting spontaneous activity, amongst other findings, which differed from those recorded in normal rats. Various studies have been conducted in this area, most of which draw similar conclusions *i. e.* increased receptive fields, reduced thresholds to mechanical, and or thermal stimulation and an increased responsiveness to stimulation. Generally, it can be demonstrated that administration of local anaesthetics after injury, or conditioning stimuli, does not alter the progression of sensitization and indicates that mechanisms other than those acting peripherally are important *i. e.* changes in the central nervous system are important (Woolf, 1989).

3.1.3 Hyperalgesia and allodynia- chemical mediators

3.1.3.1 Peripheral mechanisms

Both peripheral and central mechanisms contribute to the development and persistence of hyperalgesia and allodynia, and in the periphery, sensitization of nociceptors is an important component. Endogenous chemical mediators released during tissue damage may cause the sensitization of nociceptors, either *via* a direct, or indirect mechanism. The induction of pain by endogenous vasoactive substances including acetylcholine, 5-hydroxytryptamine (5-HT), histamine, angiotensin, substance P and bradykinin was demonstrated by Armstrong *et al.* (1952, 1953, 1957) by applying these compounds to blister bases. Noradrenaline, adrenaline and isoprenaline were shown to induce mechanical hyperalgesia in the rat paw model (Ferreira and Nakamura, 1979b) and subsequently, the role of the sympathetic nervous system in the development of inflammatory hyperalgesia was investigated (Levine *et al.* , 1986a; Nakamura and Ferreira, 1987). Although histamine, acetylcholine and 5-HT have been demonstrated to cause overt pain and hyperalgesia with associated changes in afferent sensitivity (Skouby, 1953; Rosenthal, 1964; Gilfoil and Klavins, 1965; Beck and Handwerker, 1974), bradykinin and the prostaglandins have been proposed as the most important mediators of pain and hyperalgesia associated with inflammation (Vane, 1983).

Prostaglandins are released after mechanical or chemical stimulation (Ferreira and Vane, 1967), and their presence in inflammatory exudates was demonstrated directly by Willis (1969), and confirmed in later studies (Greaves *et al.* , 1971; Velo *et al.* , 1973). Prostaglandins have been shown to induce hyperalgesia under various experimental conditions (Horton, 1963; Juhlin and Michaelsson, 1969; Willis, 1969; Karim, 1971; Collier and Schneider, 1972; Ferreira, 1972; Ferreira *et al.*, 1973; Juan and Lembeck, 1973; Moncada *et al.*, 1975; Stazewsba-Barczak *et al.*, 1976; Ferreira *et al.*, 1978a; 1978b).

The study by Ferreira (1972) was pivotal in the development of the popular theory that prostaglandins themselves are not mediators of overt pain, but cause hyperalgesia by sensitizing nociceptors to the effects of other mediators, resulting in pain. He demonstrated that PGE_1, bradykinin and histamine, when infused subdermally into human skin did not result in overt pain. However, although the simultaneous injection of histamine and bradykinin did not alter the observed response, when PGE_1 was injected with either compound alone, or both compounds together, a painful response was observed. A similar effect has been demonstrated with these mediators using a reflex increase in blood pressure as an indicator of pain after bradykinin was injected into the splenic artery (Ferreira *et al.*, 1973), the knee joint (Moncada *et al.*, 1975) or infused over the epicardium (Staszewsba-Barczak *et al.* , 1976). Further studies confirmed these findings (Collier and Schneider, 1972; Juan and Lembeck, 1973; Ferreira *et al.*, 1978b). It should be noted that Collier *et al.* (1977) reported the development of pain after administration of PGE_2, IV, in humans. Gilfoil and Klavins (1965) had noted previously that hyperalgesia did not develop immediately after injection of bradykinin, 5-HT or histamine, either on their own or in combination, in the rat paw, and had suggested that in fact these agents were exerting their effects *via* an indirect mechanism. Using different models, Guzman *et al.* (1962), Ferreira *et al.* (1973) and Moncada *et al.* (1975) all had observed similar delays between administration of these agents and their subsequent effect. It was not until 1976, that Lembeck and colleagues confirmed this hypothesis when they demonstrated that prostaglandins, primarily of the PGE group, were released after the infusion of bradykinin in an isolated perfused rabbit ear, and concluded that bradykinin augmented its algesic actions by stimulation of prostaglandin production.

Bradykinin receptors coupled to a G protein, activate phospholipase A_2 (PLA_2) to release arachidonate, and may be one of the mechanisms by which release of PGE_2 occurs (Burch and Axelrod, 1987). Studies in which PLA_2 paralleled the effects of bradykinin, and a specific PLA_2 antagonist, mepacrine, inhibited bradykinin-evoked hyperalgesia completely, (Taiwo *et al.*, 1990) supported the findings of Burch and Axelrod (1987) by demonstrating that bradykinin-evoked hyperalgesia resulted from activation of PLA_2, while the hyperalgesia seen with noradrenaline was mediated *via* PLC. However, bradykinin receptors

have been localized in thin unmyelinated nerve fibres, superficial layers of the dorsal horn of the spinal cord and small neuronal cells in sensory ganglia in guinea pigs (Steranka *et al.*, 1988), indicating that bradykinin may also exert a direct hyperalgesic effect *via* these receptors.

It is interesting to note that while arachidonic acid injected intradermally evoked a hyperalgesic response (Gonzales *et al.*, 1989), the injection of linoleic acid, also a desaturated fatty acid, did not. This suggests that the arachidonic acid exerted its effect indirectly, most probably by conversion to prostaglandins, and that the hyperalgesia observed is not a non-specific effect of desaturated fatty acids. Although, PGE_1, PGE_2 and PGI_2 all induce peripheral hyperalgesia (Ferreira, 1972; Rosenthale *et al.*, 1972; Ferreira *et al.*, 1978a), PGI_2 is a more potent hyperalgesic than PGE_2 after subplantar injection in the rat paw, although of shorter duration (Ferreira *et al.*, 1978b; Higgs *et al.*, 1978). In the latter study, PGI_2 was 5 times more potent than PGE_2 at evoking hyperalgesia while the stable metabolite of PGI_2, 6-keto-$PGF_{1\alpha}$, was not shown to be hyperalgesic. In contrast, Taiwo and Levine (1990) concluded that PGD_2 and $PGF_{2\alpha}$ did not evoke hyperalgesia in the periphery, although subsequently, PGD_2 has been shown to potentiate bradykinin-evoked hyperalgesia in guinea pigs pre-treated with a NSAID (Whelan *et al.*, 1991). In one study $PGF_{2\alpha}$ reduced the algesic action of bradykinin by antagonising the hyperalgesic actions of PGE_2 (Juan and Lembeck, 1977).

Prostaglandin-induced hyperalgesia may result from a direct action on nociceptors, but currently little evidence is available (Chahl and Iggo, 1977; Patermichelakis and Rood, 1982; Baccaglini and Hogan, 1983; Taiwo *et al.*, 1987). Prostaglandin E_2 and PGI_2 have been demonstrated in sympathetic postganglionic neurons (Gonzales *et al.*, 1989), and bradykinin, noradrenaline and leukotrienes (*vide infra*) have been shown to act indirectly to evoke hyperalgesia by stimulating the release of prostanoids from both neural and non-neural cells (Juan and Lembeck, 1974; Lembeck *et al.*, 1976; Hojvat *et al.*, 1983; Levine *et al.*, 1986a; 1986b). Interestingly, it has been suggested that bradykinin and noradrenaline induce hyperalgesia *via* different prostaglandins *i. e.* bradykinin *via* PGE_2 and noradrenaline *via* PGI_2 (Taiwo and Levine, 1988b). Taiwo *et al.* (1987), in a study designed to characterise the onset latencies of 5 different hyperalgesic agents, including PGE_2, suggested that the rapid onset of action observed for PGE_2 supported the theory that at least this prostanoid acts *via* a direct mechanism of action. In a later study, it was proposed that both PGE_2 and PGI_2 caused hyperalgesia independently of cyclooxygenase activity, peripheral adrenergic transmitters and PMNLs (Taiwo and Levine, 1989), providing further support for the theory that prostaglandins exert their effects directly.

The mechanisms by which the prostaglandins induce a cellular response is unclear. Ferreira and Nakamura (1979b) demonstrated that intraplantar injection of PGE_2, PGI_2, a stable analogue of cAMP (di-butryl cyclic adenosine monophosphate (di-butryl cAMP)), a calcium ionophore and various catecholamines which stimulate adenylate cyclase, caused a dose dependent hyperalgesia in rats pre-treated with a NSAID. Moreover, inhibition of phosphodiesterase by administration of methylxanthines, which would lead to a rise in intracellular cAMP, potentiated the evoked hyperalgesia. If hyperalgesic mediators such as the prostaglandins, induce changes in second messenger systems of afferent nerve terminals (Ferreira and Nakamura, 1979b), the sensitivity of the primary afferent may be altered by phosphorylation of membrane bound receptors (Rang and Ritchie, 1988). Repeated subplantar injection of several hyperalgesic agents, including PGE_2, at daily intervals for a period of 14 days resulted in the development of a persistent hyperalgesic state, for approximately 1 month (Ferreira *et al.* , 1990). A persistent hyperalgesia was not recorded after chronic administration of di-butryl cAMP, but instead resulted in the standard acute hyperalgesic response previously demonstrated after each administration (Ferreira and Nakamura, 1979b). Cycloheximide, an inhibitor of protein synthesis, attenuated the development of the persistent hyperalgesic response and it was suggested that the formation of a regulatory protein, controlling adenylate cyclase activation may be involved in the development of persistent hyperalgesic states. Consequently, it was proposed that prostaglandin-evoked hyperalgesia was a cAMP / calcium dependent process (Ferreira *et al.* , 1990).

The role of the leukotrienes, metabolites of the 5-lipoxygenase pathway of arachidonic acid metabolism (see chapter 1), in the induction of hyperalgesia is controversial. Originally it was thought that they did not to contribute to the hyperalgesia of inflammatory pain (Ferreira, 1981), although subsequently, this was shown not to be the case (Levine *et al.* , 1984; Bisgaard and Kristensen, 1985; Levine *et al.* , 1985a; Levine *et al.* , 1986b). Rackman and Ford-Hutchison (1983) demonstrated initially that LTD_4 evoked little hyperalgesic activity alone, but, as expected, PGE_2 augmented the effect. Subsequently, Levine *et al.* (1984; 1985a) showed that LTB_4 caused a polymorphonuclear leucocyte-(PMNL) dependent hyperalgesia which was not diminished by pre-treatment with a NSAID. Moreover, they demonstrated that PMNLs released a small molecular weight lipid factor when incubated with LTB_4, which itself evoked a maximal hyperalgesia in PMNL depleted rats. Using high performance liquid chromatography, this product demonstrated a similar retention time to a product of 15-lipoxygenation of arachidonic acid, [8R, 15S]-dihydroxyeicosa-(5E-9, 11, 13Z)-tetraenoic acid ([8R, 15S]-diHETE). In a later study, Levine *et al.* (1986b) showed [8R, 15S]-diHETE was hyperalgesic in rats, while the (8S) stereoisomer of the compound, [8S, 15S]-diHETE, not only antagonised the effects of [8R,

15S]-diHETE, but also caused a dose dependent hypoalgesia. In contrast, [8S, 15S]-diHETE did not affect PGE_2-induced hyperalgesia.

It has been suggested that (8R, 15S)-diHETE may evoke hyperalgesia *via* a direct, cyclooxygenase independent mechanism (Levine *et al.*, 1986b), and it was shown to have a similar onset latency to PGE_2 (Taiwo *et al.*, 1987). Bradykinin and noradrenaline-evoked hyperalgesia have similar onset latencies (Taiwo *et al.*, 1987), although both are cyclooxygenase dependent (Lembeck *et al.*, 1976) and rely on intact sympathetic innervation (Levine *et al.*, 1986a).

Cytokines, inflammatory proteins, have been demonstrated to induce hyperalgesia (Ferreira *et al.*, 1988; Cunha *et al.*, 1991; Cunha *et al.*, 1992). Interleukin-1 releases prostaglandins (Bernheim *et al.*, 1980) which cause a hyperalgesic response (*vide supra*), and Ferreira *et al.* (1988) demonstrated that IL-1β administered systemically in rats was a potent mechanical hyperalgesic itself, approximately 3000 times more potent than IL-1α. Moreover, they demonstrated that local pre-treatment with a NSAID attenuated the IL-1β response, indicating that the hyperalgesic effect may result from the release of prostaglandins. Interleukin-8 also has been demonstrated to cause the development of hyperalgesia in rats after intraplantar injection (Cunha *et al.*, 1991). However, neither NSAIDs, nor a specific tripeptide analogue of IL-1β which antagonised the hyperalgesic effects of IL-1β (Ferreira *et al.*, 1988), attenuated IL-8-evoked hyperalgesia. Cunha *et al.* (1991) concluded that IL-8-induced hyperalgesia was independent of both prostaglandin and IL-1β production and that hyperalgesia involved the sympathetic nervous system because the hyperalgesic effect of IL-8 was attenuated by propranolol and atenolol (β-adrenoreceptor antagonists), a dopamine receptor antagonist and guanethidine, a sympathetic neurone-blocking agent. Cunha *et al.* (1992) demonstrated that tumour necrosis factor α (TNFα) and IL-6 caused a mechanical hyperalgesia in rats and they established an order of potency for the known hyperalgesic cytokines: IL-1β>TNFα>>IL-8>>IL-6. Interleukin-6 was shown to evoke hyperalgesia *via* a common pathway with IL-1β and prostaglandin production, and indeed IL-6 may induce production of IL-1β. Although IL-6-induced production of IL-1β has not been demonstrated to date, interleukins are known to induce their own production and production of other cytokines (Dinarello *et al.*, 1986; Dinarello *et al.*, 1987; Van Damme *et al.*, 1987; Streiter *et al.*, 1989). In contrast, TNFα was found to evoke hyperalgesia *via* both the IL-1β/IL-6/prostaglandin and IL-8/sympathetic mediated hyperalgesic pathways (Cunha *et al.*, 1992).

3.1.3.2 Central mechanisms

The question still remains of how sensitization of the central nervous system occurs, and it has been addressed recently by Woolf (1989; 1991). Several neuropeptides including substance P (Lembeck *et al.* , 1977), met- and leu-enkephalin (Huges *et al.* , 1975), somatostatin (Hokfelt *et al.*, 1976), cholecystokinin (Larsson and Rehfeld, 1979) calcitonin gene related peptide (Kuraishi *et al.*, 1988) and vasoactive intestinal polypeptide (Hokfelt *et al.* , 1983) have been demonstrated in primary sensory neurons both peripherally and centrally, and a role for these agents as neurotransmitters or neuromodulators has been suggested (Hokfelt *et al.*, 1983). Urban and Randic (1984) showed that substance P and the endogenous neurotransmitter for dorsal-root-elicited slow depolarization, act in the same manner and subsequently it was demonstrated that such slow potentials could summate, producing depolarization (Thompson *et al.*, 1990). However, these findings in themselves would not explain prolonged hypersensitivity states which can occur following injury. It is possible that cellular changes including alterations in second messenger systems (cAMP, calcium *etc.*), phosphorylation of membrane receptors or ion channels or alterations in gene expression (Hunt *et al.*, 1987), occur as a result either of depolarization, or by the action of neurotransmitters. More recently, Woolf and Thompson (1991) examined the effects of a competitive N-methyl-D-aspartate (NMDA) excitatory amino acid receptor antagonist, 3((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (D-CPP), and a non-competitive antagonist, MK-801, on the establishment of both windup, a phenomenon whereby progressive increase in action potential discharge occurs after repeated stimulation (Mendell, 1984), and central sensitization. They showed not only a reduction in the facilitation of the flexor reflex in the rat by administration of the antagonists, but also demonstrated that the antagonists were capable of abolishing the hypersensitive state once it had become established. These findings were confirmed by Ren *et al.* in 1992, while Malmberg and Yaksh in the same year (1992a) used NMDA intrathecally to induce hyperalgesia in rats, and reported that pre-treatment with a selective antagonist (MK-801) abolished the response. However, administration of the antagonist after the application of NMDA did not modify the hyperalgesic response observed in the latter study.

The prostaglandins have been shown to induce hyperalgesia when administered directly onto the spinal cord. To date, the administration of PGD_2 and PGE_2 intrathecally in mice has resulted in the development of hyperalgesia while injection of $PGF_{2\alpha}$ did not (Uda *et al.*, 1990). How centrally administered prostaglandins evoke peripheral hyperalgesia remains unclear, although recent work showed that hyperalgesia induced by intrathecal administration of excitatory amino acids, and substance P, was reversed by the administration of NSAIDs, suggesting that the prostanoids may play a role in noxious

information processing at the level of the spinal cord (Malmberg and Yaksh, 1992a). It is interesting to note that NMDA receptors can activate the arachidonic acid cascade in primary culture of striatal neurons resulting in the release of arachidonic acid and 11- and 12-hydroxyeicosatetraenoic acid *via* a calcium dependent PLA_2 (Dumuis *et al.*, 1992).

3.1.4 Hyperalgesia and clinical pain

There have been many experimental studies investigating the mechanisms and effects of primary and secondary hyperalgesia, and extensive clinical studies in humans based on patient or observer report of the effect of various analgesic regimes on peri-operative pain. However, there are very few studies which have investigated the effect of clinical surgery on sensory thresholds to noxious stimulation in humans (Ekblom and Hansson, 1987; Hansson *et al.*, 1988; Lund *et al.*, 1990; Dahl *et al.*, 1992). Ekblom and Hansson (1987) demonstrated that thresholds to noxious thermal stimulation in humans were not altered in the presence of clinical pain (of up to 4 days duration), and a study in the following year confirmed these findings (Hansson *et al.*, 1988). Other workers demonstrated an increase in the perception threshold to electrical stimulation in patients after abdominal hysterectomy (Lund *et al.*, 1990), in contrast to experimental studies (Woolf, 1983). Lund and colleagues (1990) suggested that the analgesia detected post-operatively may have occurred *via* activation of descending inhibitory pathways within the central nervous system. In contrast, in patients undergoing gynaecological laparotomy, thresholds to electrical stimulation decreased while pain to suprathreshold stimulation increased post-operatively (Dahl *et al.*, 1992). In the latter study, fentanyl, a short acting μ-opioid agonist was administered at induction of anaesthesia and patients requiring post-operative analgesia were given morphine and paracetamol. Post-operative assessments were made at least 12 hours after last administration of analgesic. It is interesting that post-operative hyperalgesia was detected after administration of post-operative analgesics. There have been no similar studies carried out in the domestic species, but Ley *et al.* (1989) recently demonstrated that thresholds to noxious mechanical stimulation in sheep suffering from the chronically painful condition of footrot, were significantly lower than those recorded in normal sheep. Even after treatment, and clinical resolution of the condition, thresholds were still reduced. Thresholds to noxious thermal stimulation were not shown to be reduced in these sheep.

3.1.5 Analgesic testing of NSAIDs

A review of the commonly used laboratory methods for investigation of the analgesic and antinociceptive activity of the NSAIDs is given in chapters 1 and 4, and it is clear that in order to measure antinociceptive activity of these drugs an inflammatory stimulus must be present.

Although the mode of action of the NSAIDs (reviewed in chapter 1) is well documented, this alone does not appear to account for their clinical efficacy as analgesics in the control of acute pain (Vernimb and Hennessey, 1977; Milne and Twomey, 1980; Reid and Nolan, 1991). It was considered that either an additional mode, or modes, of action may be important. This was supported by the knowledge that carprofen, a NSAID, has been demonstrated to be a poor inhibitor of cyclooxyengase *in vitro* and yet exhibits potent antinociceptive actions *in vivo* (Strub *et al.* , 1982; Baruth *et al.* , 1985). It was also considered that the prostaglandins may be equally important centrally to the development of hyperalgesia, as they were peripherally.

The aim of the work in this chapter was to quantify the action of 2 NSAIDs, flunixin meglumine and carprofen, on thermally and mechanically-induced nociceptive responses in sheep, and to evaluate the mechanisms by which these drugs exerted their analgesic effects. This was approached in 3 ways. Firstly, the analgesic effects of flunixin meglumine and carprofen were evaluated in normal sheep using thermally and mechanically-induced physiological pain. Secondly, in 1989, Ley and colleagues had demonstrated that exposure to chronic pain from footrot (see chapter 1), reduced significantly the thresholds to noxious mechanical stimulation in affected sheep. The analgesic and antinociceptive activity of NSAIDs is generally difficult to detect in normal animals (see chapter 1 and 4) and the antinociceptive, or analgesic, activity of these drugs generally is evaluated after induction of an inflammatory focus which causes lowered thresholds to noxious stimuli. Commonly, NSAIDs return thresholds towards normal, which is accepted as a demonstration of the antinociceptive or analgesic efficacy of the drug. Consequently, it was hypothesized that administration of a NSAID, would return thresholds to noxious mechanical stimulation towards normal in sheep suffering footrot, indicating an antinociceptive effect of the drug, and allowing quantification of drug response. Finally, the mechanical and thermal thresholds to noxious stimulation were investigated in a group of sheep undergoing abdominal surgery. It was hypothesized that an acute inflammatory response would cause hyperalgesia detectable by either or both, mechanical or thermal nociceptive testing. Subsequently, the effects of flunixin meglumine and carprofen on the observed responses were investigated.

In addition, in the latter study, buprenorphine, a partial opioid agonist, was used as a drug with known analgesic activity in sheep (Nolan *et al.* , 1987c), and ketamine was administered to sheep prior to surgery, in order to 'test' the theories developed in experimental models of pain, that an NMDA receptor antagonist could prevent the development of hyperalgesia induced by an acute inflammatory stimulus.

3.2 Materials and Methods

3.2.1 Animals

A total of 90 sheep were used in this series of experiments. Husbandry conditions are outlined in section 2.1. Sheep were weighed, either the day prior to the experiment, or immediately before the experiment. Sheep undergoing surgery or general anaesthesia were fasted for 12 hours (see section 2.1).

Drugs

Five drugs were used during the course of these experiments; flunixin meglumine, carprofen, xylazine hydrochloride, ketamine hydrochloride and buprenorphine hydrochloride. The drugs were administered as described (see section 2.2).

3.2.2 Threshold testing apparatus

The apparatus used for testing thresholds to noxious mechanical and thermal stimulation is described in chapter 2 (section 2.9 and 2.10 respectively).

3.2.2.1 Mechanical nociceptive testing

Sheep were placed in a pen constructed from metal gates (approximately 100 cm wide, 200 cm long and 130 cm high). The leg unit was secured onto the forelimb, above the level of the carpus, and the control unit was placed on a table approximately 1.5 metres away from the sheep. When mechanical nociceptive testing was carried out on lame sheep, the leg unit was placed on the most severely affected forelimb. The sheep were allowed to settle in this environment for 30 to 60 minutes before beginning each experiment. The pressure stimulus was applied by activating the solenoid by squeezing the foot switch. During this time, the dial on the calibrated strain gauge amplifier indicated the rising force which was being applied to the leg of the sheep. When the sheep attempted to withdraw the limb from the stimulus by raising the limb from the ground, the control unit was switched off by releasing the pressure on the foot switch, and the reading on the dial recorded. At least 3 control, or baseline, readings were taken from each experimental sheep over a period of approximately 30 minutes, while 6 control, or baseline, readings were taken from each 'naive' sheep *i. e.* sheep which had no previous contact with the mechanical nociceptive testing equipment, over a period of approximately 30 minutes. Only the last 3 readings were used in the 'naive' sheep to calculate the control values for each experiment. Drugs were administered IV as a bolus, and the threshold to noxious mechanical stimulation was recorded thereafter. A maximum allowed force of 16

N was used in this series of experiments. At least 2 weeks were allowed to elapse between experiments on individual animals.

When mechanical nociceptive testing was carried out on sheep recovering from anaesthesia, the leg unit was not placed on the limb until the sheep could stand unaided.

3.2.2.2 Thermal nociceptive testing

Sheep were placed in a pen constructed from metal gates (approximately 100 cm wide, 200 cm long and 130 cm high). The sheep were allowed to settle in this environment for 30 to 60 minutes prior to the onset of the experiment. The ear clip of the thermal threshold testing apparatus was attached to the untagged ear and the junction box was held approximately 1.5 to 2 metres away from the sheep. An ear twitch, head shake or a slow blink of the ipsilateral eye were used to indicate a response to the application of the noxious thermal stimulus. When this occurred, a remote hand held cut-off switch was activated and the response temperature noted. Three or more control readings were taken from each sheep over a period of at least 30 minutes. Drugs were administered IV as a bolus, and the threshold to noxious thermal stimulation was recorded thereafter. The ear clip was removed from the ear between readings and no attempt was made to replace the clip in exactly the same position as previously. A ramp rate was selected such that automatic cut-off at 65°C was achieved in approximately 45 seconds. The ear was checked at intervals throughout the experiment for evidence of hyperaemia or thermal burn damage. If at any stage thermal burn damage became evident, the sheep was removed from the experiment and was not used in any further experiments until at least 1 week after the burn had completely healed. At least 2 weeks were allowed to elapse between experiments on individual animals.

When thermal nociceptive testing was carried out on sheep recovering from anaesthesia, the ear clip was not placed on the pinna until the sheep was in sternal recumbency.

3.2.3 Clinical assessment of sheep with footrot

Sheep suffering from footrot were assessed for lameness by an experienced observer, and were awarded a score on a numerical rating scale (NRS). The NRS consisted of 5 divisions represented by the numbers 0 to 4. The following description of each division was used as a guideline when scoring lameness: 0 = clinically sound; 1 = barely detectable lameness; 2 = obvious lameness; 3 = severe head nod ± resting affected foot when standing; 4 = carrying foot at trot. Lameness was assessed before noxious mechanical threshold testing.

Foot lesions were assessed visually before noxious mechanical threshold evaluation. Each foot was awarded a score on a scale of 0 to 4. The following descriptions were used in conjunction with the numerical value: 0 = no lesion or healed inactive lesion; 1 = mild interdigital dermatitis; 2 = extensive interdigital dermatitis; 3 = severe interdigital dermatitis and under-running of the horn of the heel and sole of the foot; 4 = severe interdigital dermatitis and under-running of the horn of the heel, sole and under-running of the walls of the hoof.

Each sheep suffering from footrot was awarded a total numerical score by adding the NRS score for lameness and the total of the scores awarded to each individual foot.

After noxious threshold evaluation, the sheep was turned onto its rump and the feet pared of overgrown or dead horn. Areas of infection were exposed and every attempt was made to avoid bleeding from the infected areas. All sheep with at least 1 foot given a score of 4, were given long acting penicillin intramuscularly (IM). Subsequently, sheep were herded into a foot bath containing a 10 % solution of zinc sulphate, and allowed to stand in this solution for at least 30 minutes. After this time, the sheep were moved into a concrete holding area and allowed to stand for at least 1 hour before being returned to the field or pen. Treatment was repeated at weekly intervals until the footrot lesions had healed.

3.2.4 Anaesthesia and surgery

3.2.4.1 Anaesthesia

Induction of general anaesthesia was achieved with either thiopentone sodium, 5 %, IV, or ketamine hydrochloride, 10 mg/kg, IV, given as a bolus. A bolus dose of thiopentone, 800 mg, was injected initially and incremental doses of the drug administered to permit endotracheal intubation. Where intubation failed after the administration of ketamine, anaesthesia was deepened by the administration of 4 % halothane in a 67 % nitrous oxide and 33 % oxygen mixture delivered *via* a circle system and a Hall's face mask.

Anaesthesia was maintained by the administration of 1.5 % halothane in a 33 % nitrous oxide and 67 % oxygen mixture delivered *via* a circle system with carbon dioxide absorption, and a minimum fresh gas flow rate of 3 litres per minute. Immediately after induction of anaesthesia, long acting penicillin, 2 ml, was administered IM to each sheep.

3.2.4.2 Surgery

The sheep were placed in dorsal recumbency and the caudal ventral abdominal region was clipped and cleansed in preparation for surgery. A ventral midline incision (approximately

10 cm) was made caudal to the umbilicus and the uterus was located and elevated to the wound. Embryo retrieval was accomplished by flushing the left, and subsequently the right ovarian duct. The abdominal wound was repaired using a simple continuous suture technique (catgut) in the musculature, and the skin edges were opposed by placement of surgical clips. The end of anaesthesia was regarded as the time that both halothane and nitrous oxide were removed from the inspired mixture. The surgical clips were removed 10 days post-operatively.

3.2.5 Study design

3.2.5.1 Study 1

A total of 8 experimental sheep were used in these investigations (numbers 1-8).

Five sheep (sheep numbers 1-5) were used to assess any behavioural effects of positioning the leg unit of the mechanical threshold testing apparatus, or the ear clip of the thermal threshold testing apparatus over a 10 minute period.

The repeatability of both the thermal and mechanical threshold testing apparatus in normal sheep was tested over a period of 6 hours using 8 experimental sheep. Recordings were made at hourly intervals.

Xylazine, 50 μg/kg, IV, was administered to 5 of these sheep and mechanical and thermal testing carried out over a period of up to 75 minutes.

Flunixin, 1.0 and 2.0 mg/kg, IV, carprofen, 0.7 and 4.0 mg/kg, IV, and physiological saline, 5 ml, were administered to 8 sheep in a cross-over design to assess the changes in thresholds to noxious mechanical and thermal stimulation in normal sheep over a 6 hour period. Recordings were made at half hourly intervals after drug or saline administration.

The person assessing the thresholds to noxious mechanical and thermal stimulation in this study was aware of which treatment group sheep had been allocated to.

3.2.5.2 Study 2

A total of 47 sheep were used in these investigations (numbers 1-47).

Thresholds to noxious mechanical stimulation were assessed over a 30 minute period in a group of 25 normal sheep (numbers 1-25) selected at random from a farm flock. Foot trimming was carried out on 10 of these sheep (numbers 16-25), and on the following 2 days thresholds to noxious mechanical stimulation were recorded again for these animals.

Thresholds to noxious mechanical stimulation were assessed over a 30 minute period in a group of 22 sheep (numbers 26-47) suffering from various degrees of footrot. These sheep were selected from the same flock as the 25 normal sheep, and were divided at random into 3 groups. Foot trimming was carried out on 8 of the sheep (numbers 26-33) and on the following 2 days thresholds to noxious mechanical stimulation were recorded again for these animals.

The remaining 2 groups of 7 sheep were administered flunixin meglumine, 1.0 (numbers 34-40) or 2.0 mg/kg (numbers 41-47) ,IV. Thresholds to noxious mechanical stimulation then were assessed in these sheep over a 30 minute period after drug administration. Thresholds to noxious mechanical stimulation were assessed in those sheep which received flunixin 1.0 mg/kg (numbers 34-40) again on the following 4 days. After assessment on day 2 and day 3, these sheep were injected with a repeat dose of the drug.

3.2.5.3 Study 3

Thresholds to noxious mechanical and thermal stimulation were assessed over a 30 minute period in a group of 35 sheep (numbers 1-35).

Computer generated random numbers were used to allocate 25 of these sheep, which were to undergo surgery, to 1 of 4 treatment groups. Group 1 (sheep numbers 7-12), 2 (sheep numbers 13-18) and 3 (sheep numbers 1-6) were administered the following drugs intra-operatively, IV: flunixin (1.0 mg/kg), carprofen (4.0 mg/kg) and buprenorphine (10 µg/kg), respectively. Group 4 received no treatment (sheep numbers 19-25). In the recovery period, thresholds to noxious mechanical and thermal stimulation again were assessed approximately every 30 minutes for a 2 hour period. Treatments administered in this group were done so 'blind' *i. e.* the person assessing the thresholds to noxious mechanical and thermal stimulation was unaware to which treatment group sheep had been allocated. Drugs were administered IV immediately after embryo retrieval was completed.

A further 4 sheep (numbers 26-29) were anaesthetised but did not undergo any surgical procedure or receive further treatment. In the recovery period, thresholds to noxious mechanical and thermal stimulation were assessed in these sheep over a 2 hour period.

Anaesthesia was induced by the injection of ketamine, 10 mg/kg, IV, in 6 sheep (numbers 30-35) which were to undergo surgery. In the recovery period, thresholds to noxious mechanical and thermal stimulation were assessed over a 2 hour period.

The time to sternal recumbency and to standing from the end of general anaesthesia were recorded for all sheep.

3.2.6 Statistical analyses

3.2.6.1 Study 1

Initially, distributions for thresholds were investigated for normality, using normal probability plots and correlation analysis. Analysis of variance was used to test for differences in thresholds to noxious mechanical and thermal stimulation over a 6 hour period in a group of normal experimental sheep.

A Kruskal-Wallis test, for non-parametric data, was used to test for differences over time after the administration of xylazine. Further analysis was carried out using a Wilcoxon signed rank test for non-parametric data.

Initially, distributions for thresholds were investigated for normality, using normal probability plots and correlation analysis. To test for differences in thresholds to noxious mechanical and thermal stimulation over a 6 hour period in a group of normal sheep administered test drugs or saline, a general linear model routine was used. A 3 factor nested analysis of variance provided statistical tests for drug treatment, animal and time factors and their two-way interactions.

Statistical tests were carried out using a 5% significance level.

3.2.6.2 Study 2

Initially, distributions for thresholds in all groups and subgroups of sheep were investigated for normality, using normal probability plots and correlation analysis. All groups and subgroups of sheep were normally distributed except for the group of 25 normal farm sheep.

Statistical comparisons between groups, and within groups which were normally distributed, were made using either an unpaired *t* test or a paired Student's *t* test, respectively. Comparisons between groups and within groups which were not normally distributed, were made using either a Mann-Whitney U test or a Wilcoxon signed rank test, respectively. If maximal mechanical threshold was attained by any sheep, comparisons for that group were made using tests appropriate for non-parametric data.

A oneway analysis of variance was used to test for differences between groups for total lameness scores, and for differences over time after drug administration. Further analysis was carried out using a paired Student's *t* test.

Statistical tests were carried out using a 5% significance level.

3.2.6.3 Study 3

Initially, oneway analysis of variance was used to assess differences between treatment groups for thresholds obtained pre-operatively and also at 120 minutes post-operatively. Subsequently, in view of the unbalanced study design, with differing numbers of sheep in each group and missing data, statistical analysis was undertaken using a general linear model routine. The experimental design was suitable for analysis using a 3 factor analysis of variance, previously described (see 3.2.6.1). Further analysis was carried out using a Wilcoxon signed rank test.

Statistical tests were carried out using a 5% significance level.

3.3 Results

3.3.1 Study 1

The sheep did not resent the presence of the leg unit of the mechanical threshold testing apparatus, and continued to ruminate or eat during the period of application. A similar finding occurred when the ear clip of the thermal threshold testing apparatus was attached to the sheep. However, 1 sheep immediately dislodged the ear clip by shaking its head. When the clip was repositioned, the sheep did not attempt to dislodge the clip again.

Mechanical nociceptive testing

The individual, mean ±SEM for thresholds to noxious mechanical stimulation in a group of normal sheep over a 6 hour time period are shown in Table 3.1. The individual values have a range from 1.9 N to 6.8 N, with a range of mean values from 2.5 to 3.3 N (Fig. 3.1). There were no significant changes in the thresholds to noxious mechanical stimulation over the 6 hour recording period.

The administration of xylazine caused an elevation of the threshold to noxious mechanical stimulation in all sheep (Table 3.2). Sheep numbers 3 and 5 reached maximum force level (16 N) by 15 minutes, and sheep number 1 by 30 minutes, after the administration of the drug. The remaining 2 sheep (numbers 2 and 4) achieved maximum threshold levels of 15.8 N and 12.5 N respectively, at the 15 minute reading. The time course of drug effect

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	Mean	±SEM
Time (hr)				Thermal thresholds						
0	55.0	56.2	52.0	57.8	55.7	53.2	52.2	53.9	54.5	0.72
0.5	53.4	55.6	54.2	57.3	53.5	52.9	53.1	54.7	54.3	0.53
1	54.3	55.8	55.5	54.5	55.5	50.7	51.7	53.7	54.0	0.66
2	54.5	53.7	55.9	56.5	57.0	51.7	52.1	50.5	54.0	0.85
3	56.3	54.9	50.0	52.7	56.5	51.7	54.1	52.9	53.6	0.80
4	54.1	54.1	52.4	56.2	57.4	50.7	52.3	54.8	54.0	0.77
5	55.4	55.4	54.1	55.5	56.4	55.2	52.7	53.7	54.8	0.42
6	54.6	54.9	54.9	56.4	55.7	52.8	50.8	53.4	54.2	0.63
				Mechanical thresholds						
0	2.5	3.2	2.9	2.5	2.5	6.8	2.5	2.9	3.2	0.52
0.5	2.5	2.2	3.2	2.9	1.9	4.5	2.2	2.5	2.6	0.19
1	2.9	2.5	2.9	2.5	2.2	6.5	2.9	3.2	3.2	0.48
2	2.2	2.5	3.2	2.2	3.2	4.5	2.9	2.9	3.0	0.26
3	2.5	2.5	3.9	2.5	2.2	5.8	3.2	2.5	3.1	0.43
4	2.9	3.2	3.9	2.5	2.2	5.8	3.2	2.9	3.3	0.40
5	2.5	2.2	3.2	2.9	2.2	3.2	3.2	2.9	2.8	0.15
6	2.9	2.5	3.5	2.9	3.2	3.9	3.5	2.9	3.2	0.16

Table 3.1. Individual, mean and standard error of the mean (SEM), for thresholds to noxious mechanical (Newtons) and thermal (°C) stimulation in a group of 8 normal sheep over a period of 6 hours (hr).

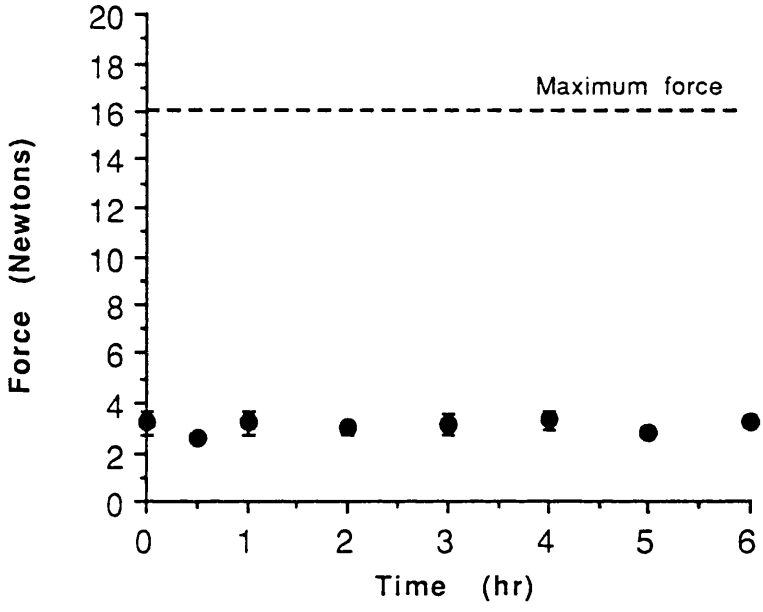

Figure 3.1. Control mechanical threshold responses in sheep recorded over a 6 hour (hr) time course.

The response threshold (Newtons) is plotted against time (hr). Each point indicates the mean and standard error of the mean for 8 sheep. The first recording was taken at time 0. The maximum threshold force (16 N) is indicated.

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	Mean	±SEM
			Thermal thresholds				
C	52.7	53.4	51.7	53.7	52.2	52.7	0.37
5	62.2	57.8	55.2	62.6	59.2	59.4	1.38
10	65.0	59.4	65.0	65.0	65.0	63.9	*
15	65.0	63.1	65.0	65.0	65.0	64.6	*
20	65.0	63.9	65.0	58.1	65.0	63.4	*
30	65.0	63.2	65.0	65.0	59.6	63.6	*
45	58.6	60.4	53.8	53.0	60.2	57.2	1.59
50	54.5	56.4	53.5	54.1	55.5	54.8	0.52
55	53.7	54.1	55.2	54.1	55.1	54.4	0.30
60	53.5	53.8	54.0	53.4	54.4	53.8	0.18
65	54.1	53.9	52.4	53.9	53.1	53.5	0.32
			Mechanical thresholds				
C	2.7	3.7	2.7	3.1	2.3	2.9	0.24
15	8.5	15.8	16	12.5	16	14.3	*
30	16	15.8	15.2	4.5	4.5	10.0	*
45	9.8	6.5	4.5	2.5	2.2	5.1	1.40
60	2.5	3.9	3.9	2.5	2.2	3.0	0.50
75	1.9	1.9	2.5	2.2	1.9	2.1	0.10

Table 3.2: Thresholds to noxious mechanical and thermal stimulation in sheep administered xylazine hydrochloride.

Individual and mean thresholds to noxious mechanical (Newtons) and thermal (°C) stimulation, ± standard error of the mean (SEM), in a group of 5 normal sheep administered xylazine hydrochloride (50 μg/kg), IV. The SEM is not shown where at least 1 sheep attained a maximal response at that time.

The control readings (C) are the mean of at least 3 thresholds recorded over a 30 minute period prior to drug administration at time 0.

*At least 1 sheep attained the maximum threshold level.

is demonstrated in Fig. 3.2. Thresholds had returned to baseline values in each sheep within 60 minutes of drug administration. Sedation was evident within 2 to 3 minutes of injection of xylazine. Two sheep remained standing throughout the study period, but the remaining 3 sheep assumed sternal recumbency approximately 5 minutes after drug administration. The sheep drooled saliva during the period of sedation and marked hyperpnoea was observed. Three of the 5 sheep began to mouth breathe within 10 minutes of drug injection. Sedation began to wane 25 minutes after xylazine injection and all sheep began ruminating between 55 and 65 minutes after drug administration. At the end of the experiment when the sheep were removed from the pen there was no evidence of ataxic gait.

The individual, mean ±SEM for thresholds to noxious mechanical stimulation in a group of normal sheep after the administration of physiological saline, 5 ml, or 1 of 4 drug treatments (flunixin 1.0 or 2.0 mg/kg, carprofen 0.7 or 4.0 mg/kg) are shown in Tables 3.3, 3.4, 3.5, 3.6 and 3.7 respectively. Sheep number 5 was removed from the study between the 3rd and 4th crossover because of illness. The administration of saline did not change the threshold to noxious mechanical stimulation over a 6 hour recording period (Fig. 3.3). Similarly, the administration of flunixin and carprofen had no effect on threshold values (Fig. 3.4 and 3.5 respectively). There were no significant differences between the 5 different treatment groups and moreover, there were no significant group x time interactions.

Thermal nociceptive testing

The individual, mean ±SEM for thresholds to noxious thermal stimulation in a group of normal sheep are shown in Table 3.1 The individual values have a range from 50.0 to 57.8 °C over the 6 hour recording period with a range of mean values from 53.6 to 54.8 °C (Fig. 3.6). There were no significant changes in the thresholds to noxious thermal stimulation over the 6 hour recording period.

Xylazine caused an elevation of the threshold to noxious thermal stimulation in all sheep (Table 3.2). Sheep numbers 1, 3, 4 and 5 reached maximum threshold level (65 °C) within 10 minutes of injection (Table 3.2). Sheep number 2 did not achieve this level at any stage after drug administration, but achieved a maximum threshold level of 63.9 °C at the 20 minute reading. The time course of drug effect is shown in Fig. 3.7. Thresholds had returned to baseline values for each sheep within 65 minutes of drug administration.

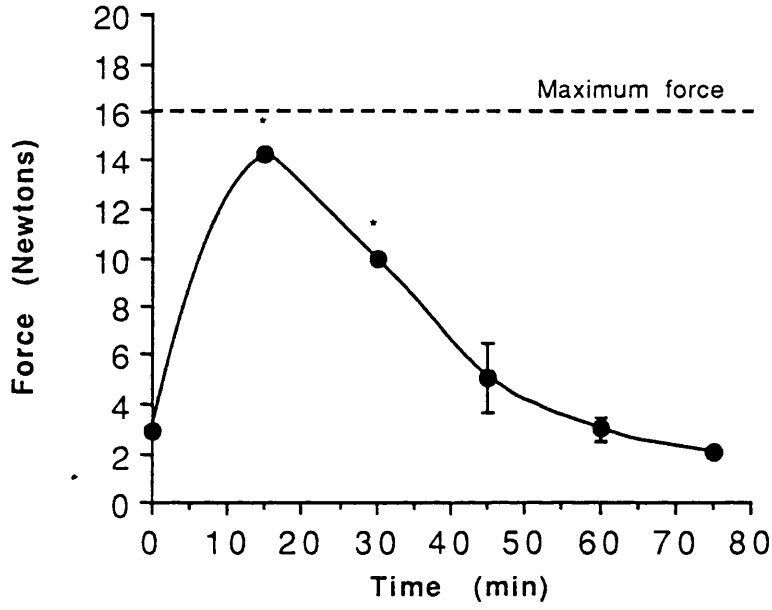

Figure 3.2. Changes in mechanical threshold responses after administration of xylazine hydrochloride (50 μg/kg, IV) in sheep.

The response threshold (Newtons) is plotted against time (min). Each point indicates the mean and standard error of the mean for 5 sheep. The SEM is not shown where at least 1 sheep attained a maximal response at that time. The pre-test reading is the mean of at least 3 readings for each of 5 sheep over a thirty minute time period before drug administration at time 0, and is shown at time 0.

The maximum threshold force (16 N) is indicated.

*$p \leq 0.05$, significantly different from pre-test values.

Time (hr)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	Mean	±SEM
					Thermal thresholds					
C	57.6	51.8	55.9	48.7	57.4	55.1	54.5	50.5	53.9	1.16
0.5	56.4	51.5	50.5	49.9	56.9	53.3	53.5	49.1	52.6	1.03
1	51.9	51.1	53.5	52.5	54.9	51.9	51.0	49.1	52.0	0.61
1.5	57.8	52.9	54.9	50.1	54.8	52.5	55.4	51.0	53.7	0.89
2	54.2	51.0	54.4	46.1	56.2	53.4	55.5	50.8	52.7	1.16
2.5	57.4	51.6	56.3	48.3	55.5	52.7	53.5	58.3	54.2	1.18
3	54.9	53.1	55.3	48.5	56.8	53.6	54.5	50.4	53.4	0.96
3.5	53.5	54.3	54.1	47.3	57.4	52.2	55.6	48.5	52.9	1.21
4	54.4	51.6	54.2	48.1	55.9	51.6	53.3	57.5	53.3	1.03
4.5	53.2	54.1	53.6	46.8	56.4	51.8	55.5	56.8	53.5	1.13
5	53.4	51.9	54.7	48.6	57.9	53.9	54.2	53.6	53.5	0.93
5.5	52.3	52.1	52.9	46.6	58.1	52.8	52.0	MD	52.4	1.18
6	53.9	52.8	54.4	48.9	55.7	51.5	53.2	MD	52.9	0.78
					Mechanical thresholds					
C	2.6	2.0	2.2	2.8	3.3	5.0	2.1	4.2	3.0	0.38
0.5	2.5	2.2	2.9	1.5	2.9	5.5	2.5	3.9	3.0	0.43
1	3.2	1.9	1.9	3.2	2.2	5.2	1.9	4.9	3.1	0.48
1.5	3.2	2.5	3.2	2.2	2.5	3.9	2.5	7.2	3.4	0.58
2	3.5	3.2	MD	2.2	1.9	3.2	1.9	4.5	2.9	0.34
2.5	2.9	2.5	3.9	2.9	1.9	3.2	1.9	4.2	2.9	0.30
3	3.9	2.9	2.5	2.5	1.9	4.2	1.9	4.5	3.0	0.36
3.5	3.2	1.9	2.9	2.5	1.9	4.5	1.9	4.5	2.9	0.39
4	3.2	3.9	3.2	2.5	1.9	3.2	1.9	4.5	3.0	0.32
4.5	3.2	2.5	3.5	2.9	1.9	3.2	2.5	3.9	3.0	0.23
5	3.2	2.9	2.9	2.5	1.9	4.5	1.9	4.2	3.0	0.34
5.5	3.2	3.2	3.2	2.5	1.9	3.9	1.9	4.5	3.0	0.32
6	3.2	3.9	2.5	2.2	1.9	4.5	2.5	4.5	3.2	0.37

Table 3.3. Thresholds to noxious mechanical and thermal stimulation in normal sheep over 6 hours, after administration of saline, IV.

Individual and mean thresholds to noxious mechanical (Newtons) and thermal (°C) stimulation, ± standard error of the mean (SEM), in a group of 8 normal sheep over a period of 6 hours (hr) after injection of saline, 5 ml, IV.

The control readings (C) are the mean of at least 3 thresholds recorded over a 30 minute period prior to drug administration at time 0.

MD= Missing data.

Time (hr)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	Mean	±SEM
					Thermal thresholds					
C	50.8	48.4	56.5	50.4	MD	50.2	50.2	58.9	53.2	0.75
0.5	58.6	47.1	57.1	50.5	MD	54.9	55.5	56.5	54.1	0.68
1	49.8	50.2	58.1	50.6	MD	54.1	52.3	56.6	54.3	0.54
1.5	57.5	53.8	55.5	51.5	MD	53.4	53.2	59.4	54.8	0.53
2	MD	54.1	55.8	55.7	MD	53.4	54.6	57.1	53.8	0.67
2.5	49.9	57.2	58.2	50.1	MD	54.0	53.8	57.6	52.9	0.66
3	50.7	53.2	57.4	49.6	MD	49.1	47.8	56.9	52.8	0.70
3.5	51.2	51.5	51.9	52.2	MD	50.9	51.2	57.2	53.0	0.74
4	52.1	49.4	58.2	59.9	MD	49.5	52.0	56.4	53.2	0.79
4.5	50.1	50.1	58.8	53.1	MD	50.5	48.3	59.1	53.0	0.76
5	50.4	49.1	58.0	51.2	MD	53.0	50.9	56.8	53.2	0.67
5.5	51.1	49.7	59.3	MD	MD	55.1	49.5	55.8	53.9	1.59
6	MD	50.3	55.9	55.0	MD	53.4	49.9	56.9	53.6	1.11
					Mechanical thresholds					
C	2.5	2.3	3.7	2.5	MD	3.7	2.3	4.5	3.1	0.19
0.5	4.8	3.2	3.5	1.9	MD	2.9	2.5	4.5	3.3	0.37
1	1.9	2.5	3.5	2.9	MD	3.2	1.9	6.5	3.2	0.56
1.5	2.5	2.5	3.9	2.5	MD	3.9	2.5	5.8	3.4	0.44
2	3.5	3.2	3.9	3.2	MD	3.2	4.5	5.2	3.8	0.27
2.5	3.2	2.5	3.9	1.9	MD	2.5	2.2	4.5	3.0	0.34
3	1.9	3.2	2.5	1.9	MD	3.2	2.9	4.5	2.9	0.32
3.5	2.9	3.5	3.5	1.9	MD	2.9	2.5	3.9	3.0	0.24
4	2.9	2.2	3.2	1.9	MD	3.9	4.5	5.2	3.4	0.43
4.5	3.9	3.2	2.9	2.5	MD	4.5	3.2	5.2	3.6	0.34
5	2.5	2.5	3.2	1.9	MD	3.2	3.2	5.2	3.1	0.37
5.5	2.5	2.5	3.5	1.9	MD	3.2	4.5	4.2	3.2	0.34
6	3.2	2.5	3.9	1.9	MD	3.2	3.9	3.9	3.2	0.28

Table 3.4. Thresholds to noxious mechanical and thermal stimulation in normal sheep over 6 hours, after administration of flunixin meglumine, 1.0 mg/kg, IV.

Individual and mean thresholds to noxious mechanical (Newtons) and thermal (°C) stimulation, ± standard error of the mean (SEM), in a group of 8 normal sheep over a period of 6 hours (hr) after injection of flunixin meglumine, 1.0 mg/kg, IV.

The control readings (C) are the mean of at least 3 thresholds recorded over a 30 minute period prior to drug administration at time 0.

MD= Missing data.

Time (hr)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	Mean	±SEM
					Thermal thresholds					
C	52.3	51.3	46.7	49.8	55.5	53.1	51.0	52.9	53.1	0.75
0.5	52.7	50.8	45.5	51.6	58.0	52.7	53.1	58.9	54.0	0.82
1	52.8	50.5	55.1	55.7	63.3	52.8	53.2	54.9	54.3	0.84
1.5	52.1	54.0	58.3	54.5	63.2	51.5	55.2	54.7	53.4	0.80
2	50.8	51.7	50.9	50.9	64.8	53.6	55.6	53.1	53.3	0.83
2.5	53.9	50.6	47.8	51.6	MD	51.9	53.0	54.5	53.0	0.75
3	52.6	53.7	47.5	50.1	63.7	54.2	55.1	54.9	51.1	2.14
3.5	56.4	51.3	45.6	51.3	56.6	54.4	54.9	53.9	50.4	2.12
4	51.0	51.2	48.3	51.2	58.5	49.8	51.6	53.4	50.2	2.10
4.5	51.5	52.3	47.9	50.8	57.8	49.8	54.4	MD	52.2	0.66
5	51.9	52.5	47.3	51.4	58.4	54.3	50.5	53.0	52.4	0.67
5.5	51.2	50.7	47.1	50.2	59.6	53.4	50.8	53.5	52.2	1.48
6	52.5	53.5	48.1	51.4	59.9	52.2	51.1	54.0	52.9	1.38
					Mechanical thresholds					
C	2.9	2.6	2.5	3.0	2.6	3.9	2.9	4.9	3.2	0.29
0.5	2.2	1.9	3.2	3.2	2.2	3.2	2.2	6.5	3.3	0.27
1	3.2	3.9	2.5	2.5	1.9	3.9	1.9	6.8	3.4	0.26
1.5	5.2	2.9	3.2	2.5	3.9	3.5	3.2	3.9	3.5	0.24
2	4.8	5.2	1.9	2.5	2.2	2.5	MD	4.8	3.2	0.24
2.5	2.5	5.8	3.2	1.9	1.9	3.9	3.9	4.5	3.2	0.26
3	2.5	2.5	3.5	1.9	1.9	2.5	3.2	3.9	2.9	0.22
3.5	5.2	2.5	3.2	1.9	2.9	2.9	2.5	6.5	3.2	0.23
4	2.2	2.5	1.9	2.5	2.5	2.9	2.5	3.2	3.0	0.17
4.5	5.2	3.9	2.9	2.5	2.9	2.9	3.5	4.8	3.3	0.19
5	4.2	3.2	2.5	2.5	2.2	2.9	2.5	3.9	3.1	0.20
5.5	3.9	3.9	2.5	1.9	2.2	2.9	3.2	5.2	3.1	0.43
6	4.5	3.5	2.9	2.2	1.9	2.9	1.9	5.2	2.9	0.44

Table 3.5. Thresholds to noxious mechanical and thermal stimulation in normal sheep over 6 hours, after administration of flunixin meglumine, 2.0 mg/kg, IV.

Individual and mean thresholds to noxious mechanical (Newtons) and thermal (°C) stimulation, ± standard error of the mean (SEM), in a group of 8 normal sheep over a period of 6 hours (hr) after injection of flunixin meglumine, 2.0 mg/kg, IV.

The control readings (C) are the mean of at least 3 thresholds recorded over a 30 minute period prior to drug administration at time 0.

MD= Missing data.

Time (hr)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	Mean	±SEM
					Thermal thresholds					
C	44.7	59.7	53.6	54.1	48.2	51.1	57.0	52.6	54.0	0.92
0.5	44.9	60.5	52.6	53.2	54.9	49.2	57.0	55.2	54.4	0.75
1	57.8	61.4	52.2	54.1	59.8	52.9	58.0	50.5	54.8	0.63
1.5	54.3	56.5	52.1	54.6	54.2	52.7	55.7	51.7	54.5	0.49
2	56.6	MD	53.5	52.3	52.2	50.1	58.4	58.3	55.0	0.62
2.5	54.0	MD	52.8	58.8	52.1	56.1	56.1	55.5	54.6	0.67
3	59.2	60.5	54.0	55.8	52.1	54.6	56.5	59.6	54.2	0.68
3.5	50.0	59.9	53.6	52.8	48.1	53.1	56.7	53.6	53.8	0.62
4	51.6	57.9	52.0	53.6	50.2	55.5	56.5	53.8	54.1	0.66
4.5	52.1	58.1	53.4	54.6	50.1	49.9	58.1	55.3	53.7	0.70
5	MD	60.4	57.3	50.0	49.9	50.9	58.2	54.3	53.4	0.70
5.5	48.3	57.5	53.8	53.3	50.2	50.2	56.0	53.9	53.6	1.03
6	47.9	58.2	52.1	54.3	51.6	51.6	56.6	52.5	53.8	1.00
					Mechanical thresholds					
C	3.8	2.3	3.0	2.0	3.0	4.3	9.8	5.9	4.3	0.90
0.5	2.9	1.9	2.9	2.9	2.5	3.2	5.8	4.5	3.4	0.23
1	3.2	1.9	3.5	1.9	3.5	4.5	3.9	4.8	3.4	0.25
1.5	3.9	1.9	3.2	2.9	2.2	3.2	3.9	5.8	3.2	0.25
2	5.2	1.9	3.2	MD	2.5	3.5	2.5	6.2	3.1	0.24
2.5	2.5	1.9	2.2	1.9	2.5	4.5	2.5	2.9	3.0	0.21
3	5.2	2.5	2.5	2.5	2.5	4.2	2.5	3.9	3.4	0.27
3.5	3.9	1.9	2.5	1.9	1.9	4.5	3.2	4.8	3.4	0.28
4	7.2	2.2	2.9	2.5	2.9	5.2	3.5	3.9	3.4	0.26
4.5	2.9	1.9	3.9	1.9	2.9	4.8	3.2	5.2	3.2	0.21
5	3.5	1.9	3.9	2.5	2.5	2.5	3.2	4.5	3.1	0.19
5.5	3.9	2.2	3.9	1.9	2.9	2.5	2.5	5.2	3.0	0.44
6	3.9	1.9	3.5	2.2	2.9	2.9	2.5	4.5	2.9	0.33

Table 3.6. Thresholds to noxious mechanical and thermal stimulation in normal sheep over 6 hours, after administration of carprofen, 0.7 mg/kg, IV.

Individual and mean thresholds to noxious mechanical (Newtons) and thermal (°C) stimulation, ± standard error of the mean (SEM), in a group of 8 normal sheep over a period of 6 hours (hr) after injection of carprofen, 0.7 mg/kg, IV.

The control readings (C) are the mean of at least 3 thresholds recorded over a 30 minute period prior to drug administration at time 0.

MD= Missing data.

Time (hr)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	Mean	±SEM
				Thermal thresholds						
C	52.8	49.9	49.8	57.9	MD	50.2	50.2	44.6	51.8	0.69
0.5	52.1	55.1	51.9	55.6	MD	54.6	52.5	47.8	52.1	0.65
1	51.6	50.4	55.9	56.0	MD	51.2	51.8	45.8	51.2	0.64
1.5	55.3	49.5	55.3	55.2	MD	50.1	49.7	46.2	50.9	0.58
2	50.7	49.6	49.2	MD	MD	52.9	50.8	46.6	51.3	0.64
2.5	53.5	50.1	52.3	MD	MD	49.4	53.1	46.7	51.7	0.70
3	54.1	48.8	55.1	56.4	MD	57.1	51.9	46.6	52.1	0.74
3.5	54.2	49.6	51.4	57.1	MD	49.5	50.7	45.9	51.5	0.71
4	54.1	49.4	50.7	58.2	MD	51.6	54.6	46.9	51.5	0.69
4.5	52.9	50.3	50.3	55.6	MD	47.8	54.3	45.7	51.4	0.58
5	52.4	50.5	51.6	56.2	MD	47.5	53.2	46.6	52.0	0.54
5.5	50.5	52.0	51.9	54.8	MD	51.2	53.2	51.1	52.4	0.53
6	50.4	51.5	51.5	55.1	MD	56.9	54.6	48.6	53.0	1.15
				Mechanical thresholds						
C	3.2	3.3	3.1	2.2	MD	3.2	2.0	3.7	3.0	0.23
0.5	3.2	1.9	2.2	1.9	MD	3.2	3.2	3.9	3.1	0.24
1	2.5	2.5	2.5	2.5	MD	3.2	1.9	6.4	3.2	0.26
1.5	3.9	2.5	2.5	5.8	MD	2.5	3.2	3.9	3.2	0.23
2	2.5	1.9	3.2	2.9	MD	2.5	5.8	3.2	3.2	0.21
2.5	3.2	1.9	2.9	1.9	MD	3.2	4.5	3.9	3.2	0.20
3	3.9	3.2	2.9	2.5	MD	2.5	2.5	5.2	3.4	0.21
3.5	3.2	3.2	4.5	1.9	MD	2.5	2.9	5.2	3.5	0.21
4	3.9	2.9	4.5	2.5	MD	2.9	3.9	5.5	3.4	0.21
4.5	3.9	2.9	3.5	1.9	MD	3.5	2.5	4.8	3.2	0.18
5	2.9	2.9	3.9	1.2	MD	3.9	3.9	4.5	3.2	0.17
5.5	3.2	2.9	3.2	1.9	MD	2.5	3.2	3.9	2.9	0.26
6	2.9	3.2	3.2	1.9	MD	3.9	3.9	4.5	3.4	0.34

Table 3.7. Thresholds to noxious mechanical and thermal stimulation in normal sheep over 6 hours, after administration of carprofen, 4.0 mg/kg, IV.

Individual and mean thresholds to noxious mechanical (Newtons) and thermal (°C) stimulation, ± standard error of the mean (SEM), in a group of 8 normal sheep over a period of 6 hours (hr) after injection of carprofen, 4.0 mg/kg, IV.

The control readings (C) are the mean of at least 3 thresholds recorded over a 30 minute period prior to drug administration at time 0.

MD= Missing data.

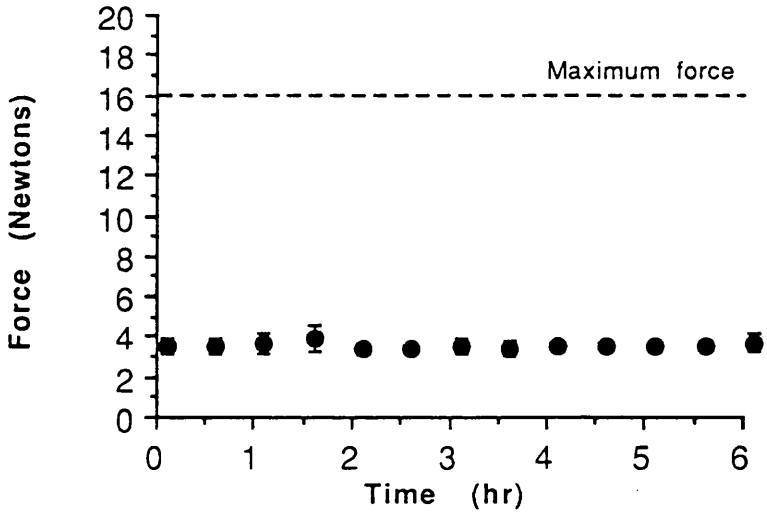

Figure 3.3. Changes in mechanical threshold responses after the administration of saline (5 ml, IV) in 8 sheep.

The response threshold (Newtons) is plotted against time (hr). Each point indicates the mean and standard error of the mean for 8 sheep. The pre-test reading is the mean of at least 3 readings for each sheep over a thirty minute time period before drug administration at time 0, and is shown at time 0.

The maximum threshold force (16 N) is indicated.

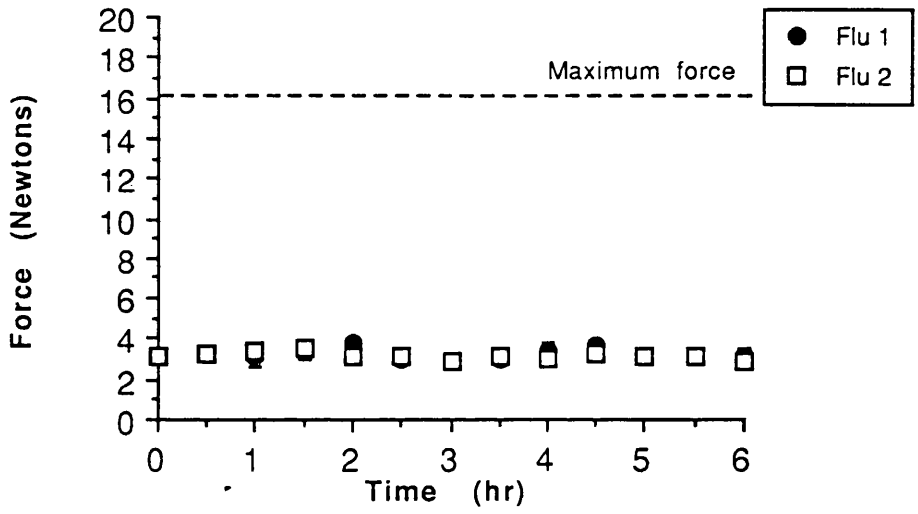

Figure 3.4. Changes in mechanical threshold responses after the administration of flunixin meglumine, 1.0 (Flu 1) and 2.0 (Flu 2) mg/kg, IV, in sheep.

The response threshold (Newtons) is plotted against time (hr). Each point indicates the mean and standard error of the mean for 7 (Flu 1) or 8 (Flu 2) sheep. The pre-test reading is the mean of at least 3 readings for each sheep over a thirty minute time period before drug administration at time 0, and is shown at time 0.

The maximum threshold force (16 N) is indicated.

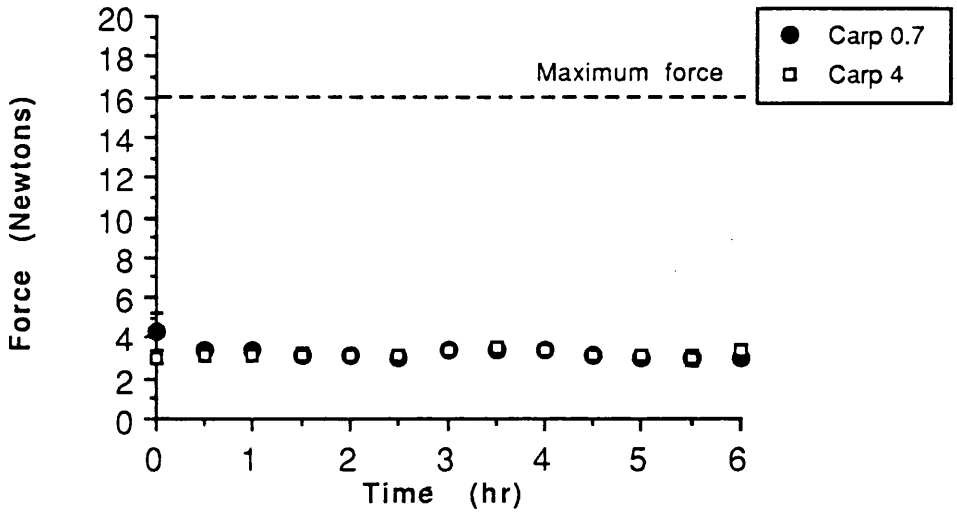

Figure 3.5. Changes in mechanical threshold responses after the administration of carprofen, 0.7 (Carp 0.7) and 4.0 (Carp 4) mg/kg, IV, in sheep.

The response threshold (Newtons) is plotted against time (hr). Each point indicates the mean and standard error of the mean for 7 (Carp 4) or 8 (Carp 0.7) sheep. The pre-test reading is the mean of at least 3 readings for each sheep over a thirty minute time period before drug administration at time 0, and is shown at time 0.

The maximum threshold force (16 N) is indicated.

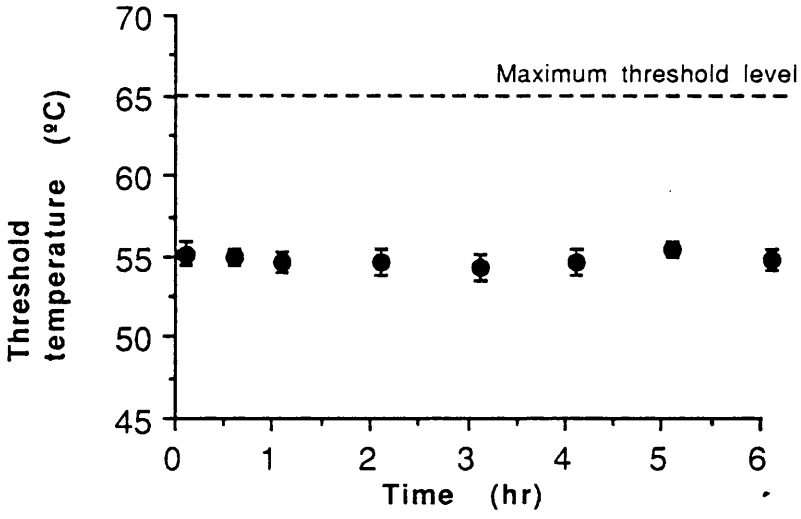

Figure 3.6. Control thermal threshold responses in sheep recorded over a 6 hour (hr) time course.

The response threshold (°C) is plotted against time (hours). Each point indicates the mean and standard error of the mean for 8 sheep. The first recording was taken at time 0.

The maximum threshold temperature (65°C) is indicated.

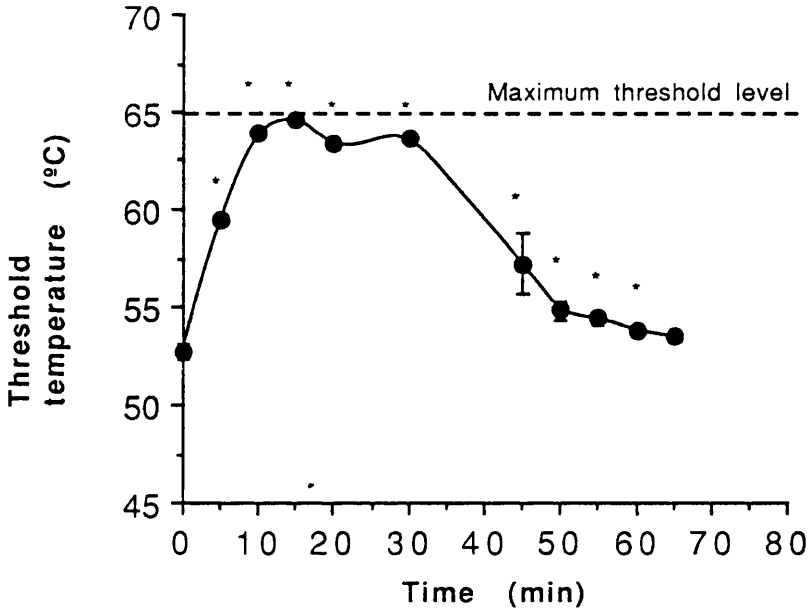

Figure 3.7. Changes in thermal threshold responses after administration of xylazine hydrochloride (50 μg/kg, IV) in sheep.

The response threshold (°C) is plotted against time (min). Each point indicates the mean and standard error (SEM) of the mean for 5 sheep. The SEM for each time point is not shown where at least 1 sheep attained a maximal response at that time. The pre-test reading is the mean of at least 3 readings for each of 5 sheep over a thirty minute time period before drug administration and is shown at time 0.

The maximum threshold temperature (65°C) is indicated.

*$p \leq 0.05$, significantly different from pre-test values.

The individual, mean ±SEM for thresholds to noxious thermal stimulation in a group of normal sheep after the administration of physiological saline, 5 ml, or 1 of 4 drug treatments (flunixin 1.0 or 2.0 mg/kg, carprofen 0.7 or 4.0 mg/kg) are shown in Tables 3.3, 3.4, 3.5, 3.6 and 3.7 respectively. The administration of saline did not change the threshold to noxious thermal stimulation over the 6 hour recording period (Fig. 3.8).

Flunixin appeared to raise the mean thermal threshold values slightly, from 0.5 hours after administration of 1.0 or 2.0 mg/kg, to 2.0 hours after injection (Fig. 3.9). Similarly, carprofen, 0.7 and 4.0 mg/kg, IV, appeared to raise and lower, respectively, the mean threshold to noxious thermal stimulation from 0.5 hours after drug injection to 3 hours post injection (Fig. 3.10). However, statistical analysis of the thermal threshold data indicated no significant differences between the 5 different treatment groups and no significant group x time interactions.

3.3.2 Study 2

Thresholds to noxious thermal stimulation were not determined in this study.

Mechanical nociceptive testing

Thresholds to noxious mechanical stimulation were recorded for a group of 25 normal sheep selected at random from a farm flock. The individual and mean thresholds for each of these sheep, taken over a 30 minute period, are shown in Table 3.8. Statistical analysis of this data indicated that the thresholds to noxious mechanical stimulation in these 'naive' sheep (group mean, 4.9N) were significantly different ($p \leq 0.01$) from the control threshold values obtained in a group of 8 experimental sheep tested routinely (group mean, 3.0N) (Table 3.3).

Ten sheep (numbers 16-25) (Table 3.8) were selected at random and after mechanical threshold testing on day 1, had their feet pared and were allowed to stand in a foot bath for at least 30 minutes. Threshold to noxious mechanical stimulation were re-evaluated on the 2 following days over a similar time course (Table 3.9). Statistical analysis of the data indicated that there were no significant differences between the readings obtained on day 1 (group mean, 4.7N) and those obtained on day 2 (group mean, 4.0N), but the values recorded on day 1 were significantly greater ($p \leq 0.05$) than those recorded on day 3 (group mean, 3.1N) for the same sheep. Mechanical thresholds for the larger group of sheep (n=25) (Table 3.8) on day 1 were also significantly greater ($p \leq 0.01$) than those recorded on day 3 for the smaller group (n=10). Moreover, it is interesting to note that there no significant differences between the thresholds recorded on day 3 (group mean,

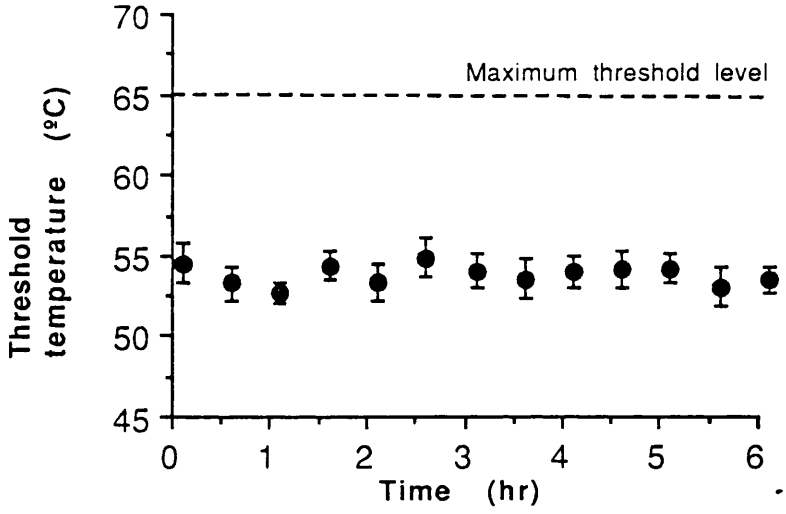

Figure 3.8. Changes in thermal threshold responses after administration of saline (5 ml, IV) in sheep.

The response threshold (°C) is plotted against time (hr). Each point indicates the mean and standard error of the mean for 8 sheep. The pre-test reading is the mean of at least 3 readings for each sheep over a thirty minute time period before drug administration and is shown at time 0.

The maximum threshold temperature (65°C) is indicated.

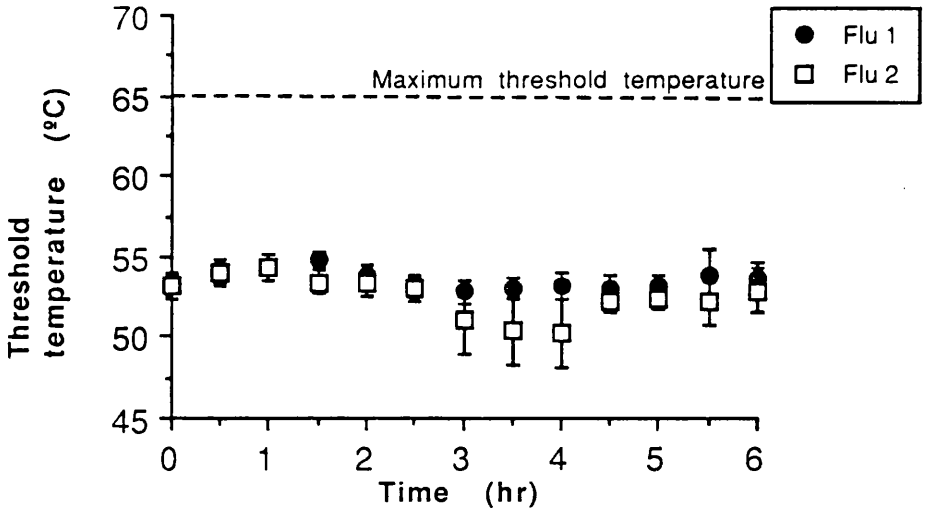

Figure 3.9. Changes in thermal threshold responses after administration of flunixin meglumine 1.0 (Flu 1) and 2.0 (Flu 2) mg/kg, IV, in sheep.

The response threshold (°C) is plotted against time (hr). Each point indicates the mean and standard error of the mean for 8 sheep. The pre-test reading is the mean of at least 3 readings for each sheep over a thirty minute time period before drug administration and is shown at time 0.

The maximum threshold temperature (65°C) is indicated.

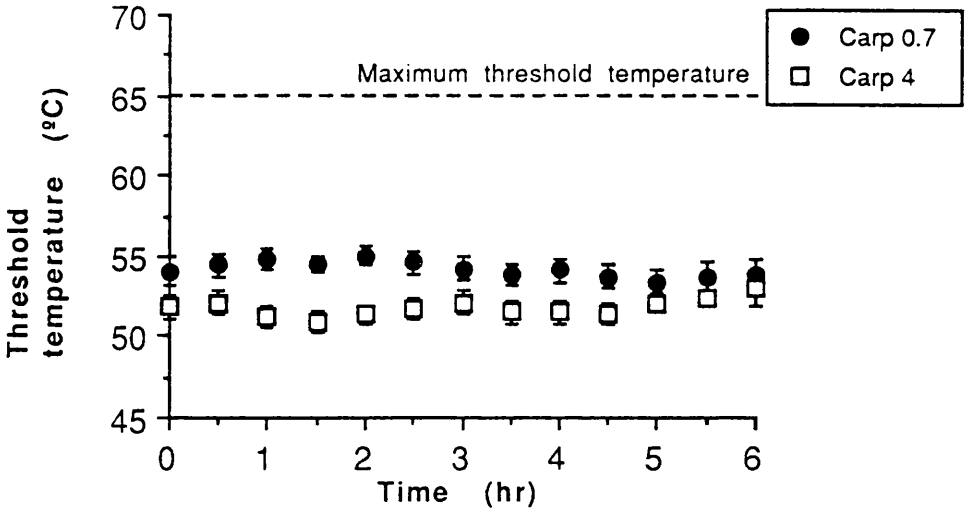

Figure 3.10. Changes in thermal threshold responses after administration of carprofen 0.7 (Carp 0.7) and 4.0 (Carp 4) mg/kg, IV, in sheep.

The response threshold (°C) is plotted against time (hr). Each point indicates the mean and standard error of the mean for 8 sheep. The pre-test reading is the mean of at least 3 readings for each over a thirty minute time period before drug administration and is shown at time 0.

The maximum threshold temperature (65°C) is indicated.

Sheep Number	T1	T2	T3	Mean
1	3.2	3.5	5.5	4.1
2	5.2	4.5	5.2	5.0
3	3.9	4.5	4.2	4.2
4	6.8	5.5	5.2	5.8
5	3.5	3.2	3.5	3.4
6	8.5	9.8	8.5	8.9
7	2.5	2.2	4.2	3.0
8	5.2	3.9	3.9	4.3
9	5.2	4.5	4.5	4.7
10	3.2	2.5	2.5	2.7
11	5.8	5.8	5.5	5.7
12	3.5	2.9	2.9	3.1
13	13.2	12.5	11.2	12.3
14	4.5	3.2	4.2	4.0
15	3.9	3.5	4.5	4.0
16	5.8	5.8	5.8	5.8
17	2.9	2.5	2.5	2.6
18	3.5	3.9	3.9	3.8
19	3.9	4.2	3.9	4.0
20	3.9	4.5	3.9	4.6
21	3.2	3.9	3.9	3.7
22	7.8	7.8	7.8	7.8
23	6.5	5.8	6.2	6.2
24	3.2	3.2	3.2	3.2
25	5.2	5.2	4.8	5.1

Table 3.8. Thresholds to noxious mechanical stimulation in farm sheep unaccustomed to the testing procedure.

Thresholds to noxious mechanical stimulation (Newtons) in a group of 25 normal sheep selected at random from a farm flock. Thresholds (T1 -T3) were recorded over a 30 minute period.

	No. 16	No. 17	No. 18	No. 19	No. 20	No. 21	No. 22	No. 23	No. 24	No. 25
					Day 1					
T 1	5.8	2.9	3.5	3.9	3.9	3.2	7.8	6.5	3.2	5.2
T 2	5.8	2.5	3.9	4.2	4.5	3.9	7.8	5.8	3.2	4.8
T 3	5.8	2.5	3.9	3.9	3.9	3.9	7.8	6.2	3.2	5.2
Mean	5.8	2.6	3.8	4.0	4.6	3.7	7.8	6.2	3.2	5.1
					Day 2					
T 1	3.2	2.5	3.9	3.9	5.5	4.2	4.5	3.9	3.2	3.2
T 2	2.5	1.9	4.2	4.2	10.5	3.9	4.5	3.9	3.2	3.9
T 3	3.2	2.5	3.9	3.9	8.2	3.9	4.5	3.9	3.2	3.2
Mean	3.0	2.3	4.0	4.0	8.1	4.0	4.5	3.9	3.2	3.4
					Day 3					
T 1	2.9	3.2	3.9	2.5	4.2	2.5	1.5	3.9	4.2	1.5
T 2	2.5	3.5	2.9	2.5	4.5	2.5	1.5	3.9	4.5	1.9
T 3	2.9	3.2	3.5	2.2	4.5	2.5	1.9	3.9	5.2	1.9
Mean	2.8	3.3	3.4	2.4	4.4	2.5	1.6	3.9	4.6	1.8

Table 3.9. Thresholds to noxious mechanical stimulation in farm sheep unaccustomed to the testing procedure, over a period of 3 days.

Thresholds to noxious mechanical stimulation (Newtons) in a group of 10 normal sheep selected at random from a farm flock. Thresholds (T1 -T3) were recorded over a 30 minute period on 3 sequential days. The feet of all sheep were pared after threshold readings on day 1.

3.1N) and those recorded in a group of 8 experimental sheep over a similar time course (group mean, 3.0N) (Table 3.3).

Thresholds to noxious mechanical stimulation in 22 sheep suffering varying degrees of foot rot (group mean, 5.1N) (Table 3.10) were not significantly different from the control thresholds obtained on day 1 for a group of 25 normal sheep from the same farm (group mean, 4.7N) (Table 3.8). A summary of the lameness scores and the foot lesion scores for the 22 sheep are shown in Table 3.11. There were no significant differences between the total scores for lameness (group mean, sheep numbers 26-33, 2.13; 34-40, 1.86; 41-47, 2.29) and foot lesions (group mean, sheep numbers 26-33, 4.13; 34-40, 4.14; 41-47, 3.86) awarded to the sheep in the 3 groups to which these animals were allocated.

Eight of these sheep (sheep numbers 26-33) were selected at random and after mechanical threshold testing on day 1, had their feet pared and were allowed to stand in a foot bath for at least 30 minutes. Subsequently, thresholds to noxious mechanical stimulation in these 8 sheep were determined as on day 1 on the 2 following days (Table 3.12). Statistical analysis of the data indicated no significant change in the threshold to noxious mechanical stimulation over the 3 day test period for these sheep (group mean day 1, 3.8N; day 2, 4.6N; day 3, 4.6N). In addition, the thresholds recorded on day 3 were not significantly different from the thresholds recorded for a group of normal sheep (n=25) from the same farm (group mean 4.7N) (Table 3.8), but were still found to be significantly different from those thresholds recorded in a group of experimental sheep tested routinely (group mean, 3.0N) (Table 3.3).

The individual and mean thresholds to noxious mechanical stimulation in sheep suffering from footrot were determined after the administration of flunixin meglumine at 1.0 and 2.0 mg/kg (sheep numbers 34-40 and 41-47 respectively) (Table 3.13a and 3.13b respectively). There were no significant treatment x time interactions within each group. However, there was a significant difference between the 2 treatment groups. Detailed examination of the data shows that the mean pre-treatment threshold values obtained for sheep given flunixin, 1.0 mg/kg, (7.6 N) (Table 3.13a) are at least twice those found for sheep administered flunixin, 2.0 mg/kg, (3.6 N) (Table 3.13b), thus accounting for the recorded differences (Fig. 3.11). Thresholds to noxious mechanical stimulation were recorded in 4 of the 7 sheep administered flunixin, 2.0 mg/kg, IV, up to 120 minutes after drug administration (data not shown), and no changes from pre-treatment values were observed.

Sheep Number	T1	T2	T3	Mean
26	2.2	1.5	2.2	2.0
27	4.5	10.5	5.8	6.9
28	2.5	3.2	2.9	2.9
29	4.8	5.2	4.8	4.9
30	3.2	4.8	3.9	4.1
31	2.9	2.2	2.2	2.4
32	2.5	2.9	3.2	2.9
33	5.2	3.2	4.2	4.2
34	9.5	6.2	6.2	7.3
35	4.5	4.5	4.5	4.5
36	7.2	7.2	6.8	7.1
37	6.5	6.8	6.2	6.5
38	11.8	12.2	11.8	11.9
39	5.8	7.2	7.8	6.9
40	9.2	7.2	11.2	9.2
41	2.5	1.9	2.5	2.3
42	5.2	4.5	5.5	5.1
43	2.2	1.9	2.2	2.1
44	2.5	2.5	2.5	2.5
45	3.5	3.2	3.5	3.4
46	6.5	6.5	6.2	6.4
47	3.5	3.9	3.5	3.6

Table 3.10: Thresholds to noxious mechanical stimulation in farm sheep suffering from footrot and unaccustomed to the testing procedure.

Thresholds to noxious mechanical stimulation (Newtons) in a group of 22 sheep from a farm flock, suffering from varying degrees of footrot. Thresholds (T1 -T3) were recorded over a 30 minute period.

Sheep Number	NRS	Foot Score	Total Score
26	2	4	6
27	3	2	5*
28	3	9	12
29	1	1	2*
30	2	10	12
31	1	1	2*
32	1	2	3
33	4	4	8*
34	3	4	7*
35	3	5	8
36	2	6	8
37	2	2	4*
38	1	5	6
39	1	3	4*
40	1	4	5*
41	1	4	5*
42	3	3	6
43	1	3	4*
44	1	2	3
45	2	7	9
46	4	4	8
47	4	4	8

Table 3.11. Lameness and foot lesion scores in a group of sheep suffering from footrot.

Numerical rating scale (NRS) scores (0-4) for lameness, total score for foot lesions (0-4) for each foot (maximum possible score of 16) and the total of both scores combined for a group of 22 farm sheep suffering from footrot.

*Footrot only present in 1 forefoot.

	No. 26	No. 27	No. 28	No. 29	No. 30	No. 31	No. 32	No. 33
				Day 1				
T 1	2.2	4.5	2.5	4.8	3.9	2.9	3.2	5.2
T 2	1.5	10.5	3.2	5.2	4.8	2.2	2.9	3.2
T 3	2.2	5.8	2.9	4.8	3.2	2.2	2.5	4.2
Mean	2.0	6.9	2.9	4.9	4.1	2.4	2.9	4.2
				Day 2				
T 1	2.5	6.5	3.2	5.5	6.8	2.2	6.5	5.2
T 2	2.2	6.5	3.5	4.5	5.2	2.2	6.9	5.8
T 3	2.2	4.5	3.2	5.2	5.2	2.9	6.9	5.2
Mean	2.3	5.8	3.3	5.1	5.7	2.4	6.8	5.4
				Day 3				
T 1	3.5	6.2	6.5	2.9	3.9	4.2	5.2	3.2
T 2	2.9	6.5	7.2	3.2	4.8	3.9	5.2	2.5
T 3	3.5	6.5	9.2	2.9	4.8	3.9	4.8	2.9
Mean	3.3	6.4	7.6	3.0	4.5	4.0	5.1	2.9

Table 3.12. Thresholds to noxious mechanical stimulation in farm sheep suffering from footrot and unaccustomed to the testing procedure, over a period of 3 days.

Thresholds to noxious mechanical stimulation (Newtons) in a group of 8 sheep, from a farm flock, suffering from varying degrees of footrot. Thresholds (T1 -T3) were recorded over a 30 minute period on 3 sequential days. The feet of all sheep were pared after threshold readings on day 1.

Time (min)	No. 34	No. 35	No. 36	No. 37	No. 38	No. 39	No. 40	Mean	±SEM
C	7.3	4.5	7.1	6.5	11.9	6.9	9.2	7.6	0.88
1	11.8	4.8	12.2	5.2	7.2	9.8	5.8	8.1	1.18
5	8.8	5.8	8.2	5.8	9.8	7.2	11.8	8.2	0.82
9	8.5	3.2	9.2	6.2	15.5	6.8	9.2	8.4	1.43
11	8.2	5.2	9.2	6.8	16.0	7.5	9.5	8.9	*
15	10.5	9.2	6.5	6.8	16.0	8.5	8.5	9.4	*
20	9.5	8.8	6.5	6.2	16.0	9.2	5.8	8.9	*
30	7.8	7.5	9.5	6.5	16.0	9.2	6.5	7.9	*

Table 3.13a. Thresholds to noxious mechanical stimulation in sheep suffering footrot after injection of flunixin meglumine, 1.0 mg/kg, IV.

Individual and mean thresholds to noxious mechanical (Newtons) stimulation, ± standard error of the mean (SEM), in a group of 7 sheep suffering from varying degrees of footrot administered flunixin meglumine, 1.0 mg/kg, IV. The SEM is not shown where at least 1 sheep attained a maximal response at that time.

The control readings (C) are the mean of at least 3 thresholds recorded over a 30 minute period prior to drug administration at time 0.

*At least 1 sheep attained the maximum threshold level.

Time (min)	No. 41	No. 42	No. 43	No. 44	No. 45	No. 46	No. 47	Mean	±SEM
C	2.3	5.1	2.1	2.5	3.4	6.4	3.6	3.6	0.60
1	5.2	5.8	3.5	1.9	4.5	7.8	3.4	4.6	0.73
5	5.2	5.8	3.9	2.5	3.9	6.5	6.5	4.9	0.61
9	1.9	5.2	3.9	2.5	3.5	9.8	6.8	4.8	1.05
11	4.5	5.5	3.2	2.9	3.2	8.8	5.2	4.8	0.76
15	4.5	6.5	3.2	2.9	3.2	9.5	5.2	5.0	0.90
20	4.5	5.5	3.2	3.5	3.5	9.8	5.5	5.1	0.87
30	5.2	5.5	3.2	3.5	3.2	9.5	4.8	5.0	0.84

Table 3.13b. Thresholds to noxious mechanical stimulation in sheep suffering footrot after injection of flunixin meglumine, 2.0 mg/kg, IV.

Individual and mean thresholds to noxious mechanical (Newtons) stimulation, ± standard error of the mean (SEM), in a group of 7 sheep suffering from varying degrees of footrot administered flunixin meglumine, 1.0 mg/kg, IV.

The control readings (C) are the mean of at least 3 thresholds recorded over a 30 minute period prior to drug administration at time 0.

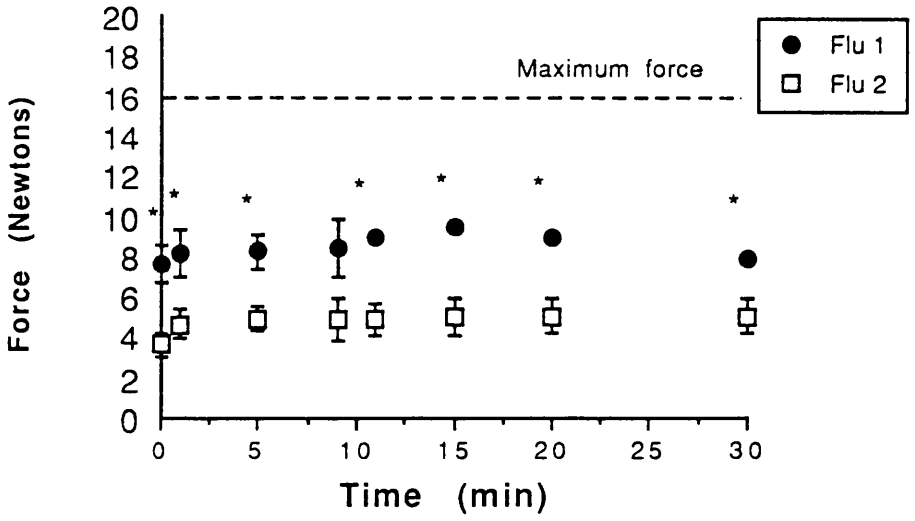

Figure 3.11. Changes in mechanical threshold responses after administration of flunixin meglumine, 1.0 (Flu 1) and 2.0 (Flu 2) mg/kg, IV, in sheep suffering footrot.

The response threshold (Newtons) is plotted against time (min). Each point indicates the mean and standard error of the mean (SEM) for 7 sheep. The SEM is not shown where at least 1 sheep attained maximal response at that time. The pre-test reading is the mean of at least 3 readings for each sheep over a thirty minute time period before drug administration at time 0, and is shown at time 0.

The maximum threshold force (16 N) is indicated.

*$p \leq 0.05$, significant difference between groups.

Thresholds to noxious mechanical stimulation in sheep suffering from footrot which received flunixin treatment over a period of 3 days were evaluated (Table 3.14). Thresholds in this group changed significantly over a 4 day period. Thresholds recorded on day 1 prior to drug treatment (group mean, 7.6N) were significantly lower than those recorded on day 2 (group mean, 10.7N) ($p \leq 0.05$), but were significantly greater than those recorded on day 3 (group mean, 6.6N) ($p \leq 0.01$) and day 4 (group mean, 6.0N) ($p \leq 0.01$). There was no significant difference between thresholds recorded on day 2 and day 3, or day 3 and day 4. On day 4, all animals except sheep number 40, had lower mean thresholds than those recorded on day 1. Thresholds recorded on day 4 were not significantly different from those recorded in a group of control sheep from the same farm on day 1 (Table 3.9) (group mean, 4.7N), and nor were they significantly different from those recorded in the same group of control sheep on day 3 (group mean, 3.1N).

3.3.3 Study 3

There were no significant differences in either the demographic distribution of sheep allocated to each treatment group in this study (Table 3.15a and 3.15b), or between groups. The mean duration of anaesthesia for each group is shown in Table 3.15a and 3.15b and was not found to be significantly different. The mean time to assume sternal recumbency was significantly longer for sheep which received buprenorphine (sheep numbers 1-6) ($p \leq 0.01$) than for all other groups, and similarly, these sheep also took a significantly longer time to stand ($p \leq 0.01$).

Mechanical nociceptive testing

The changes in threshold to noxious mechanical stimulation were recorded for all sheep in the recovery period and are documented in Tables 3.16, 3.17, 3.18, 3.19, 3.20 and 3.21. Statistical analysis of the data indicated that there were no significant differences between groups pre-operatively. The mean thresholds to noxious mechanical stimulation over a 2 hour period post-operatively are shown in Fig. 3.12. It is interesting to note that the mean thresholds to noxious mechanical stimulation for sheep given no treatment, those which did not undergo surgery, and those which received carprofen, buprenorphine or ketamine were higher 2 hours post-operatively or post-anaesthesia than control values (6.6 *vs.* 7.0, 7.1 *vs.* 9.9, 6.5 *vs.* 8.0, 5.7 *vs.* 8.2 and 4.9 *vs.* 6.5 Newtons respectively). Only those sheep which were given flunixin did not show this change (7.6 *vs.* 6.4N). However, there were no significant differences between groups post-operatively and moreover, there were no significant group x time interactions. This indicated that neither general anaesthesia alone, general anaesthesia and surgery nor general anaesthesia and surgery with drug treatment altered thresholds to noxious mechanical stimulation.

	No. 34	No. 35	No. 36	No. 37	No. 38	No. 39	No. 40
				Day 1			
T 1	9.5	4.5	7.2	6.5	11.8	5.8	9.2
T 2	6.2	4.5	7.2	6.9	12.2	7.2	7.2
T 3	6.2	4.5	6.9	6.2	11.8	7.8	11.2
Mean	7.3	4.5	7.1	6.5	11.9	6.9	9.2
				Day 2			
T 1	6.5	5.5	11.2	9.8	12.8	14.5	8.2
T 2	7.2	5.5	13.2	11.5	13.2	13.2	15.8
T 3	6.2	5.8	11.2	8.8	13.2	16.0	15.2
Mean	6.6	5.6	11.9	10.0	13.1	14.6	13.1
				Day 3			
T 1	6.8	3.9	3.2	5.2	5.8	8.5	12.5
T 2	5.2	3.5	4.5	5.8	5.5	11.2	10.8
T 3	5.8	4.2	2.9	6.2	5.8	10.5	10.5
Mean	5.9	3.9	3.5	5.7	5.7	10.1	11.3
				Day 4			
T 1	3.9	3.2	6.5	6.2	3.5	6.5	12.8
T 2	3.9	3.9	5.2	6.2	3.5	6.8	8.5
T 3	3.5	3.5	6.8	6.5	2.9	5.2	14.2
Mean	3.8	3.5	6.2	6.3	3.3	6.2	11.8

Table 3.14. Thresholds to noxious mechanical stimulation in a group of sheep suffering footrot. Response to injection of flunixin meglumine (1.0 mg/kg), IV, over a period of 4 days.

Thresholds to noxious mechanical stimulation (Newtons) in a group of 7 sheep suffering from footrot selected from a farm flock. Thresholds (T1 -T3) were recorded over a 30 minute period on 4 sequential days. The feet of all sheep were pared after threshold readings on day 1. Flunixin meglumine, 1.0 mg/kg, was administered IV after threshold evaluation on days 1, 2 and 3.

Sheep Number	Weight (kg)	Dose Thio. (mg)	Time GA (min)	Sternal (min)	Standing (min)
1	53.0	900	25	24	54
2	58.5	1000	24	47	77
3	58.0	900	20	54	79
4	49.0	900	17	51	68
5	56.0	900	21	22	49
6	64.0	1000	15	29	88
Mean	56.4	933.3	20.3	37.8*	69.2*
7	53.0	900	19	30	46
8	52.0	900	24	11	51
9	55.0	900	24	12	59
10	48.0	900	18	24	46
11	63.0	900	22	18	30
12	56.0	900	18	11	25
Mean	54.5	900	20.8	17.7	42.8
13	56.0	900	20	21	28
14	51.0	900	20	26	51
15	44.0	900	22	38	82
16	53.0	900	24	52	MD
17	56.0	900	21	16	31
18	49.0	900	19	21	54
Mean	51.5	900	21.0	29.0	49.2
19	54.0	900	21	18	40
20	54.0	900	23	18	47
21	45.0	800	20	13	23
22	59.0	900	21	14	30
23	55.0	900	15	21	39
24	53.0	900	16	24	29
25	53.0	1000	29	45	69
Mean	53.3	900	19.3	21.9	39.6

Table 3.15a. Demographic and anaesthetic details of a group of sheep undergoing general anaesthesia with or without ventral midline laparotomy.

Weight, dose of intravenous anaesthetic agent (thiopentone (Thio)) in milligrams (mg), duration of general anaesthesia (Time GA) in minutes (min), time to sternal recumbency from the end of anaesthesia (Sternal) and time to standing from the end of anaesthesia in a group of sheep undergoing ventral midline laparotomy for embryo retrieval. Group 1 (sheep numbers 7-12), 2 (sheep numbers 13-18) and 3 (sheep numbers 1-6) were administered the following drugs intra-operatively, IV: flunixin meglumine (1.0 mg/kg), carprofen (4.0 mg/kg) and buprenorphine (10 μg/kg) respectively. Group 4 (sheep numbers 19-25) received no treatment.

MD= Missing data

*$p \leq 0.01$, significant difference between groups.

Sheep Number	Weight (kg)	Dose Thio. (mg)	Time GA (min)	Sternal (min)	Standing (min)
26	64.0	1000	20	8	15
27	59.0	1000	20	15	38
28	64.0	1000	20	24	46
29	55.0	1000	20	13	25
Mean	60.5	1000	20.0	15.0	31.0
		Dose Ket. (mg)			
30	59.0	590	17	30	73
31	51.0	510	18	10	16
32	51.0	510	21	15	17
33	56.0	560	29	8	10
34	43.0	430	21	20	24
35	53.0	530	13	16	26
Mean	52.2	521.7	19.8	16.5	27.7

Table 3.15b. Demographic and anaesthetic details of a group of sheep undergoing general anaesthesia with or without ventral midline laparotomy.

Weight, dose of intravenous anaesthetic agent (thiopentone (Thio) or ketamine (Ket)) in milligrams (mg), duration of general anaesthesia (Time GA) in minutes (min), time to sternal recumbency from the end of anaesthesia (Sternal) and time to standing from the end of anaesthesia in a group of sheep undergoing ventral midline laparotomy for embryo retrieval (sheep numbers 30-35), or general anaesthesia (sheep numbers 26-29).

MD= Missing data

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Mean	±SEM
				Therm				
Pre	60.2	46.4	56.6	60.2	57.0	58.7	56.5	2.12
30	55.0	MD	MD	MD	65.0	56.9	59.0	*
45	54.0	54.0	MD	MD	59.8	59.0	56.7	1.57
60	53.0	MD	65.0	60.5	51.0	54.4	56.8	*
90	MD	47.5	60.0	MD	54.7	46.3	52.1	3.2
120	58.0	51.0	55.0	64.5	52.9	56.7	56.4	2.0
				Mech				
Pre	4.8	4.8	5.2	7.8	4.4	7.3	5.7	0.59
60	6.2	MD	MD	MD	MD	MD	6.2	0
90	7.5	3.9	6.5	8.2	7.8	8.2	7.0	0.67
120	7.8	4.2	9.8	12.8	2.5	12.2	8.2	1.72

Table 3.16. Thresholds to noxious mechanical and thermal stimulation post-operatively, for sheep administered buprenorphine (10 μg/kg, IV) intra-operatively.

Individual and mean thresholds to noxious mechanical (Mech) (Newtons) and thermal (Therm) (°C) stimulation, ± standard error of the mean (SEM), in a group of 6 normal sheep post-operatively. The SEM is not shown where at least 1 sheep attained a maximal response at that time. Buprenorphine hydrochloride, 10 μg/kg, IV, was administered within 5 minutes of the end of anaesthesia. After surgery, mechanical thresholds were evaluated once sheep were standing unaided, while thresholds to noxious thermal stimulation were evaluated once sheep were in sternal recumbency.

Pre-readings (Pre) are the mean of at least 3 thresholds recorded over a 30 minute period prior to anaesthesia.

MD= Missing data *At least 1 sheep achieved maximum threshold

Time (min)	No. 7	No. 8	No. 9	No. 10	No. 11	No. 12	Mean	±SEM
				Therm				
Pre	54.0	52.5	59.0	60.0	58.9	61.7	57.7	1.47
30	MD	51.0	58.0	65.0	57.3	62.0	58.7	*
45	55.0	48.0	62.5	59.0	54.2	64.0	57.1	2.42
60	55.0	51.0	57.0	65.0	53.9	65.0	57.8	*
90	54.0	53.0	55.0	62.0	50.9	65.0	56.7	*
120	65.0	65.0	51.0	60.0	60.7	64.0	61.0	*
				Mech				
Pre	13.5	9.2	5.3	6.0	4.0	7.7	7.6	1.39
60	13.2	8.5	11.2	9.2	5.8	7.8	9.2	1.06
90	MD	6.5	6.2	MD	10.8	MD	7.8	1.49
120	7.2	8.2	6.2	6.2	4.5	5.8	6.4	0.51

Table 3.17. Thresholds to noxious mechanical and thermal stimulation post-operatively, for sheep administered flunixin meglumine (1.0 mg/kg, IV) intra-operatively.

Individual and mean thresholds to noxious mechanical (Mech) (Newtons) and thermal (Therm) (°C) stimulation, ± standard error of the mean (SEM), in a group of 6 normal sheep post-operatively. The SEM is not shown where at least 1 sheep attained a maximal response at that time. Flunixin meglumine, 1.0 mg/kg, IV, was administered within 5 minutes of the end of anaesthesia. After surgery, mechanical thresholds were evaluated once sheep were standing unaided, while thresholds to noxious thermal stimulation were evaluated once sheep were in sternal recumbency.

Pre-readings (Pre) are the mean of at least 3 thresholds recorded over a 30 minute period prior to anaesthesia.

MD= Missing data *At least 1 sheep achieved maximum threshold

Time (min)	No. 13	No. 14	No. 15	No. 16	No. 17	No. 18	Mean	±SEM
				Therm				
Pre	56.5	56.0	58.0	59.0	54.9	57.7	57.0	0.61
30	54.0	57.0	MD	MD	51.8	65.0	57.0	*
45	61.0	52.0	64.0	MD	65.0	65.0	61.4	*
60	63.0	61.0	64.0	65.0	59.4	65.0	62.9	*
90	63.0	64.0	63.0	65.0	65.0	55.4	62.6	*
120	MD	65.0	61.0	65.0	57.1	61.0	61.8	*
				Mech				
Pre	6.8	6.4	7.6	6.2	5.4	6.8	6.5	0.30
60	10.5	8.8	MD	6.8	4.5	12.8	8.7	1.44
90	MD	MD	10.5	MD	7.8	5.2	7.8	1.53
120	6.8	9.5	4.5	MD	7.2	11.8	8.0	1.24

Table 3.18. Thresholds to noxious mechanical and thermal stimulation post-operatively, for sheep administered carprofen (4.0 mg/kg, IV) intra-operatively.

Individual and mean thresholds to noxious mechanical (Mech) (Newtons) and thermal (Therm) (°C) stimulation, ± standard error of the mean (SEM), in a group of 6 normal sheep post-operatively. The SEM is not shown where at least 1 sheep attained a maximal response at that time. Carprofen, 4.0 mg/kg, IV, was administered within 5 minutes of the end of anaesthesia. After surgery, mechanical thresholds were evaluated once sheep were standing unaided, while thresholds to noxious thermal stimulation were evaluated once sheep were in sternal recumbency.

Pre-readings (Pre) are the mean of at least 3 thresholds recorded over a 30 minute period prior to anaesthesia.

MD= Missing data *At least 1 sheep achieved maximum threshold

Time (min)	No. 19	No. 20	No. 21	No. 22	No. 23	No. 24	No. 25	Mean	±SEM
				Therm					
Pre	63.7	57.6	62.5	60.5	62.3	53.0	52.4	58.8	1.75
30	45.0	52.6	48.0	62.0	56.0	MD	45.1	51.5	2.75
45	45.5	48.0	55.0	MD	54.0	49.7	45.8	49.7	1.66
60	53.0	51.0	49.0	59.0	54.5	45.6	47.5	51.4	1.72
90	MD	55.0	49.0	60.0	64.0	51.8	47.8	54.6	2.60
120	55.5	57.0	59.0	54.0	65.0	48.1	45.5	54.9	*
				Mech					
Pre	9.6	7.0	6.2	4.8	6.8	4.0	7.5	6.6	0.69
60	14.8	10.2	11.8	7.8	9.8	3.2	MD	9.6	1.60
90	7.2	11.5	9.2	MD	7.8	4.2	5.5	7.6	1.06
120	5.8	7.8	4.5	16.0	8.2	1.2	5.8	7.0	*

Table 3.19. Thresholds to noxious mechanical and thermal stimulation for sheep post-operatively.

Individual and mean thresholds to noxious mechanical (Mech) (Newtons) and thermal (Therm) (°C) stimulation, ± standard error of the mean (SEM), in a group of 7 normal sheep post-operatively. The SEM is not shown where at least 1 sheep attained a maximal response at that time. After surgery, mechanical thresholds were evaluated once the sheep were standing unaided, while the thresholds to noxious thermal stimulation were evaluated once the sheep was in sternal recumbency.

Pre-readings (Pre) are the mean of at least 3 thresholds recorded over a 30 minute period prior to anaesthesia.

MD= Missing data *At least 1 sheep achieved maximum threshold

Time (min)	No. 26	No. 27	No. 28	No. 29	Mean	±SEM
		Therm				
Pre	54.4	51.0	54.0	51.3	52.7	0.89
30	55.4	52.4	51.8	55.3	53.7	0.95
45	55.1	58.0	55.7	55.3	56.0	0.67
60	54.6	54.0	57.1	51.9	54.4	1.07
90	51.9	57.3	53.5	51.9	53.7	1.27
120	51.6	50.0	50.3	53.4	51.3	0.77
		Mech				
Pre	2.9	9.2	10.6	5.6	7.1	1.75
60	3.9	8.5	9.8	10.2	8.1	1.45
90	2.5	8.5	9.2	13.2	8.4	2.21
120	3.9	7.5	12.2	16.0	9.9	*

Table 3.20. Thresholds to noxious mechanical and thermal stimulation for sheep post-general anaesthesia.

Individual and mean thresholds to noxious mechanical (Mech) (Newtons) and thermal (Therm) (°C) stimulation, ± standard error of the mean (SEM), in a group of 4 normal sheep post-anaesthesia. The SEM is not shown where at least 1 sheep attained a maximal response at that time. After anaesthesia, mechanical thresholds were evaluated once sheep were standing unaided, while thresholds to noxious thermal stimulation were evaluated once sheep were in sternal recumbency.

Pre-readings (Pre) are the mean of at least 3 thresholds recorded over a 30 minute period prior to anaesthesia.

*At least 1 sheep achieved maximum threshold

Time (min)	No. 30	No. 31	No. 32	No. 33	No. 34	No. 35	Mean	±SEM
				Therm				
Pre	54.4	57.4	60.1	55.9	57.9	52.1	56.3	1.14
30	57.9	MD	58.0	51.3	61.7	54.0	56.6	1.79
45	56.8	MD	59.3	57.8	61.7	54.0	57.9	1.28
60	53.9	59.3	61.3	59.0	58.9	54.1	57.8	1.24
90	56.7	42.0	56.3	53.8	63.2	56.7	54.6	2.77
120	51.0	57.8	55.5	59.3	61.3	51.3	56.0	1.73
				Mech				
Pre	2.7	6.4	3.6	8.3	5.4	2.9	4.9	0.91
60	6.2	7.8	11.2	15.2	9.2	4.5	9.0	1.56
90	3.5	8.2	MD	4.8	7.8	5.5	6.0	0.89
120	8.8	6.2	4.8	3.5	6.2	9.2	6.5	0.91

Table 3.21. Thresholds to noxious mechanical and thermal stimulation for sheep post-operatively.

Individual and mean thresholds to noxious mechanical (Mech) (Newtons) and thermal (Therm) (°C) stimulation, ± standard error of the mean (SEM), in a group of 6 normal sheep post-anaesthesia. Anaesthesia was induced by injection of ketamine hydrochloride, 10 mg/kg, IV. After surgery mechanical thresholds were evaluated once sheep were standing unaided, while thresholds to noxious thermal stimulation were evaluated once sheep were in sternal recumbency.

Pre-readings (Pre) are the mean of at least 3 thresholds recorded over a 30 minute period prior to anaesthesia.

MD= Missing data

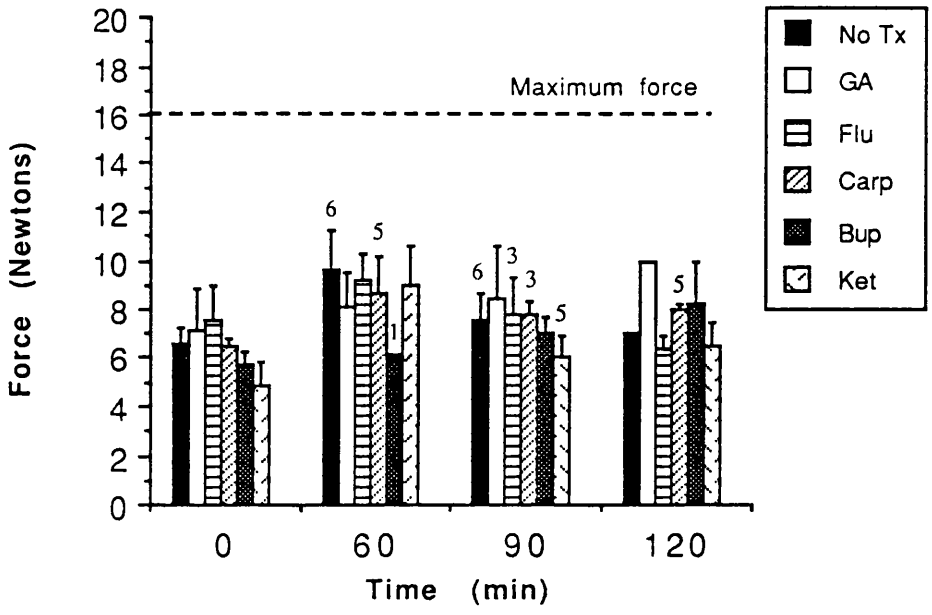

Figure 3.12. Thresholds to noxious mechanical stimulation for sheep either post-operatively and post-anaesthesia, and the effect of various intra-operative treatments.

Changes in mechanical threshold responses in 5 groups of sheep post-operatively and 1 group of sheep post-anaesthesia (n=4) (GA). Sheep which underwent surgery were allocated to the following groups: thiopentone induction (n=7) (NoTx); thiopentone induction and flunixin meglumine, 1.0 mg/kg, IV, before end of anaesthesia (n=6) (Flu); thiopentone induction and carprofen, 4.0 mg/kg, IV, before end of anaesthesia (n=6) (Carp); thiopentone induction and buprenorphine hydrochloride, 10 μg/kg, IV, before end of anaesthesia (n=6) (Bup); ketamine induction (n=6) (Ket).

The response threshold (Newtons) is plotted against time (min). Each point indicates the mean and standard error of the mean (SEM) for readings obtained at each time point. The SEM for each time point is not shown where at least 1 sheep attained a maximal response at that time. The pre-test reading is the mean of at least 3 readings for each sheep over a thirty minute time period before anaesthesia, and is shown at time time 0.

The maximum force (16N) is indicated.

All groups n=6 except No Tx, n=7, GA, n=4, unless stated below.

1- (n=1) 3- (n=3)

5- (n=5) 6- (n=6)

Thermal nociceptive testing

The changes in threshold to noxious thermal stimulation were recorded for all sheep in the recovery period and are documented in Tables 3.16, 3.17, 3.18, 3.19 3.20 and 3.21. There were no significant differences between groups pre-operatively. Further analysis indicated that there were significant differences between the groups post-operatively.

Thresholds to noxious thermal stimulation in sheep which underwent a surgical procedure, but did not receive any drug treatment, were found to be significantly lower ($p \leq 0.05$) 45, 60 and 120 minutes after the end of anaesthesia, than the thresholds obtained pre-operatively (Fig. 3.13). Sheep which were anaesthetised but did not undergo surgery, were found to have thresholds similar to those obtained pre-anaesthesia at all time points (Fig. 3.13). Thresholds to noxious thermal stimulation in sheep which underwent surgery and received flunixin prior to the end of general anaesthesia were not significantly different from those recorded pre-operatively (Fig. 3.14). Administration of carprofen yielded a similar result (Fig. 3.14). However, thresholds to noxious thermal stimulation in these sheep were significantly greater than control values for the group ($p \leq 0.05$) 60 minutes post-operatively. Thresholds to noxious thermal stimulation in sheep which underwent surgery and received buprenorphine prior to the end of general anaesthesia were not significantly different from those recorded pre-operatively (Fig. 3.15) and similarly, thresholds for sheep in which anaesthesia was induced by the administration of ketamine did not change significantly in the post-operative period (Fig. 3.16).

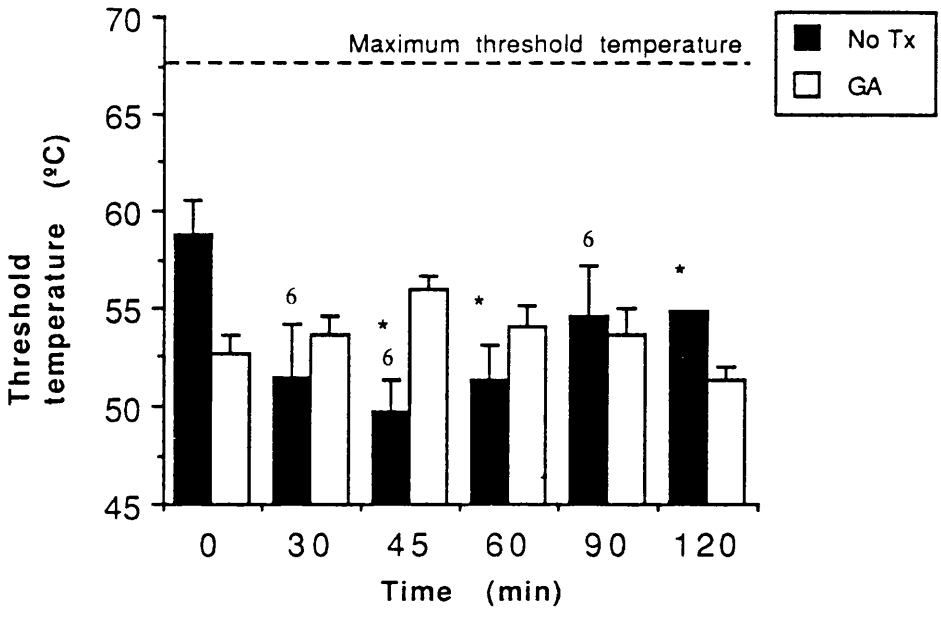

Figure 3.13. Changes in thermal threshold responses in 1 group of sheep post-operatively (n=7) (No Tx) and a second group of sheep (n=4) after general anaesthesia (GA).

The response threshold (°C) is plotted against time (min). Each point indicates the mean and standard error of the mean (SEM) for readings obtained at each time point. The SEM for each time point is not shown where at least 1 sheep attained a maximal response at that time. The pre-test reading is the mean of at least 3 readings for each sheep over a thirty minute time period before anaesthesia, and is shown at time time 0.

The maximum threshold temperature (65 °C) is indicated.

*$p \leq 0.05$, significantly different from pre-test values.

No Tx, n=7 and GA, n=4, unless stated below.

6- (n=6)

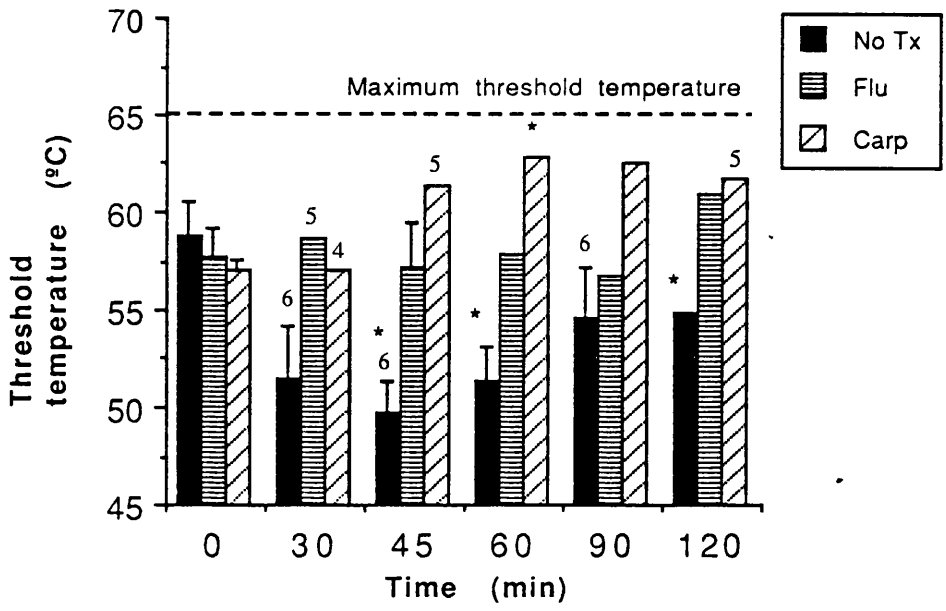

Figure 3.14. Post-operative changes in thermal threshold responses in 3 groups of sheep, 2 of which received different intra-operative treatments.

One group received no treatment (n=7) (No Tx), a second was administered flunixin meglumine (n=6) (Flu), 1.0 mg/kg, IV, within 5 minutes of the end of anaesthesia, while the third group (n=6) received carprofen, 4 mg/kg, IV.

The response threshold (°C) is plotted against time (min). Each point indicates the mean and standard error of the mean (SEM) for readings obtained at each time point. The SEM for each time point is not shown where at least 1 sheep attained a maximal response at that time. The pre-test reading is the mean of at least 3 readings for each sheep over a thirty minute time period before anaesthesia, and is shown at time time 0.

The maximum threshold temperature (65 °C) is indicated.

*p≤0.05, significantly different from pre-test values.

All groups n=6 except No Tx, n=7, unless stated below.

4- (n=4) 5- (n=5)

6- (n=6)

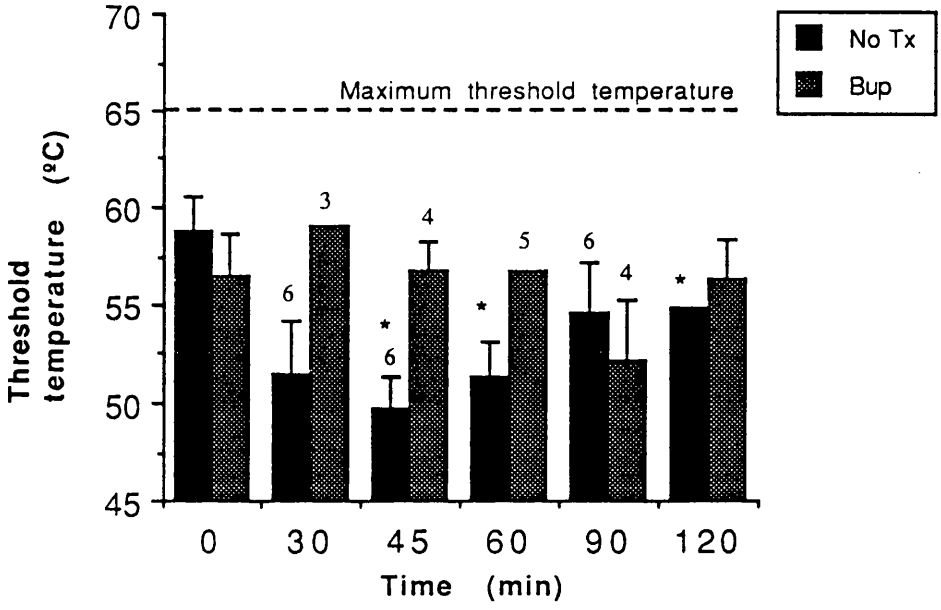

Figure 3.15. Post-operative changes in thermal threshold responses in 2 groups of sheep, 1 of which received buprenorphine hydrochloride intra-operatively.

One group received no treatment (n=7) (No Tx), the second was administered buprenorphine hydrochloride (n=6) (Bup), 10 μg/kg, IV, within 5 minutes of the end of anaesthesia.

The response threshold (°C) is plotted against time (min). Each point indicates the mean and standard error of the mean (SEM) for readings obtained at each time point. The SEM for each time point is not shown where at least 1 sheep attained a maximal response at that time. The pre-test reading is the mean of at least 3 readings for each sheep over a thirty minute time period before anaesthesia, and is shown at time time 0.

The maximum threshold temperature (65 °C) is indicated.

*$p \leq 0.05$, significantly different from pre-test values.

No Tx, n=7 and Bup, n=6, unless stated below.

3- (n=3) 4- (n=4)

5- (n=5) 6- (n=6)

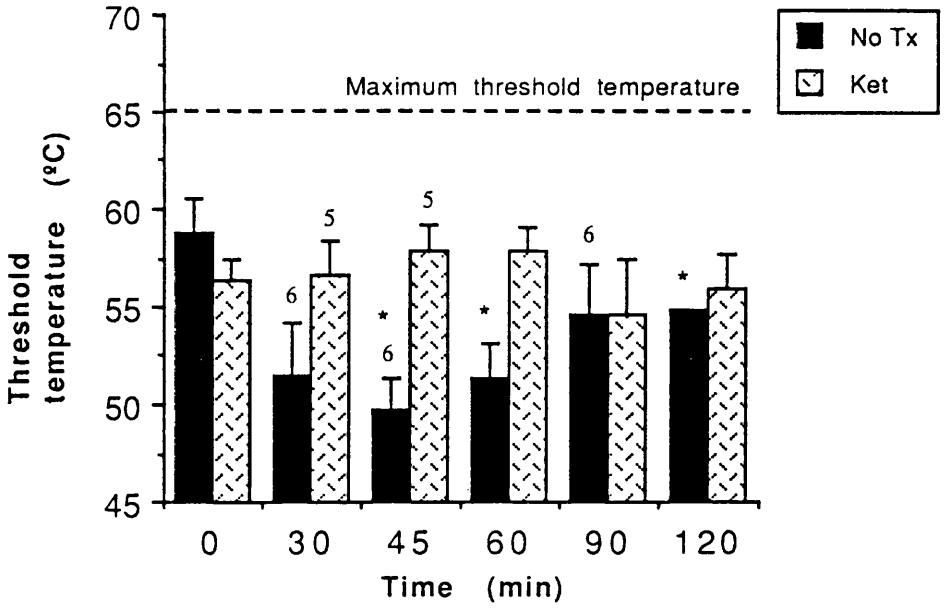

Figure 3.16. Post-operative changes in thermal threshold responses in 2 groups of sheep after induction of general anaesthesia using different agents.

Induction of general anaesthesia was achieved by administration of thiopentone, IV, in one group (n=7) (No Tx),while in the second group (n=6) (Ket), induction of general anaesthesia was achieved by administration of ketamine, IV.

The response threshold (°C) is plotted against time (min). Each point indicates the mean and standard error of the mean (SEM) for readings obtained at each time point. The SEM for each time point is not shown where at least 1 sheep attained a maximal response at that time. The pre-test reading is the mean of at least 3 readings for each sheep over a thirty minute time period before anaesthesia, and is shown at time time 0.

The maximum threshold temperature (65 °C) is indicated.

*$p \leq 0.05$, significantly different from pre-test values.

No Tx, n=7 and Ket, n=6, unless stated below.

5- (n=5) 6- (n=6)

3.4 Discussion

It has been suggested that it is important to match groups of experimental animals when examining the pharmacological effects of drugs (Ley *et al.* , 1990a), and in all 3 of the present studies sheep were matched for both breed and weight. In study 1, a total of 8 experimental sheep were used. Five of these sheep were Suffolk x Scottish Blackface, and the remaining 3 were Border Leicester x Scottish Blackface (Greyface). These sheep were closely matched for weight at the time of each experiment, and statistical analysis indicated that there were no significant differences between threshold readings obtained for individual sheep within each treatment group. There were no breed differences between sheep allocated to treatment groups in study 2 or 3, and all sheep were closely matched for weight. Variation in the analgesic effect of xylazine hydrochloride, 50 μg/kg, administered to sheep of different breeds has been observed (Ley *et al.* , 1990a). Variation in the sedative effects of this drug also have been noted in different breeds of cattle (Raptopoulos and Weaver, 1984) and between individual sheep (Kyles *et al.* , 1993), although no direct correlation with sheep breed was demonstrated in the latter study. Ley *et al.* (1990a) commented that the differences seen in their study may have reflected a breed difference, although further investigations indicated that the difference lay in bodyweight difference.

3.4.1 Study 1

The mechanical threshold testing apparatus used in this study was similar to that which had been used successfully to evaluate thresholds to noxious mechanical stimulation in horses (Chambers *et al.* , 1990; Chambers *et al.* , 1993a), and subsequently in sheep (Muge and Livingston, 1992). The thermal threshold testing apparatus was based on an original design described by Nolan *et al.* (1987a). Therefore, it was considered important that the repeatability of these 2 units was studied. Results consistent with those found previously in sheep (Nolan *et al.* , 1987a) were recorded, with no significant change in the threshold to noxious mechanical and thermal stimulation over a 6 hour period. Thresholds to both noxious mechanical and thermal stimulation also were similar to those reported previously by Nolan *et al.* (1987c). It is important that sensitization of the stimulated area does not develop over the testing period as this would influence results recorded after drug administration. Moreover, the presence of leg and ear units did not appear to alter the behaviour of the sheep used in this study. Dummy units, as previously described (Nolan *et al.* , 1987a), were not used.

Results recorded following xylazine injection, a drug with known antinociceptive properties in sheep (Nolan *et al.* , 1987a; 1987b; Ley *et al.* , 1990a; 1991a; Muge and Livingston, 1992), significantly elevated thresholds to both mechanical and thermal noxious stimulation.

Sheep were not moved after administration of xylazine, but those which remained standing swayed, and all sheep were sedated heavily. The end-points used for the mechanical and thermal threshold testing procedures used in this study are motor responses *i. e.* leg lift and ear flick. Drugs which impair motor ability *e. g.* xylazine, may therefore influence these test systems. The effects of a sedative / ataractic drug, acetylpromazine, has been investigated on the thresholds to noxious mechanical and thermal stimulation in sheep, and was found not to change thresholds significantly from pre-treatment values (Nolan *et al.* , 1987a). Similar results have been obtained in the horse (Chambers *et al.* , 1990; Chambers *et al.* , 1993a). Further investigations on the effects of non-analgesic sedative / ataractic drugs were not conducted in the present study because there is no available evidence which suggests that NSAIDs impair motor ability.

Non-steroidal anti-inflammatory drugs may be evaluated in the laboratory using a wide variety of models (Otterness and Bliven, 1985). However, laboratory models which induce inflammation *e. g.* Randall Selitto assay (Randall and Selitto, 1957), are used most commonly in the assessment of antinociceptive or analgesic efficacy of these drugs. Tests of physiological pain in normal animals frequently do not detect antinociceptive activity of NSAIDs (Winder, 1959). However, detailed inspection of thresholds to noxious thermal stimulation after administration of carprofen, 0.7 mg/kg, IV, indicated an elevation in thresholds, although not significant, which persisted for a period of approximately 2 hours, that was not evident after administration of placebo. The antinociceptive effects of carprofen have been determined in normal horses using thermal threshold testing apparatus (Schatzmann *et al.* , 1990) consisting of a heating element fixed to a girth and tightly apposed to a clipped area behind the elbow. Carprofen, 0.7 mg/kg, IV, elevated thresholds to noxious thermal stimulation in all horses tested over 24 hours. However, statistical analysis was not carried out on the carprofen data and analgesia was defined as an elevation in thermal threshold of $\geq 1°C$ at 3 successive determination times for each horse when compared with the pre-treatment values obtained for the same horse. Similar criteria applied to the data obtained in the present study for normal sheep in the first 2 hours after drug or saline administration, indicated that saline caused analgesia in one sheep, while carprofen, using either dose rate, caused analgesia in a total of 2 animals in each case. Schatzmann *et al.* (1990) also investigated the relationship between plasma concentration of carprofen and analgesia to noxious thermal stimulation. They suggested that plasma levels of carprofen ≥ 1.5 µg/ml were required to demonstrate an analgesic effect of the drug. In sheep, similar levels of carprofen have been detected in plasma for at least 72 and 48 hours after administration of 4.0 and 0.7 mg/kg, IV, respectively (Welsh *et al.* , 1992). However since analgesia was not detected in the present study it is impossible to predict if a similar plasma level of carprofen is required in sheep to produce analgesia. Unfortunately, Schatzmann *et al.* (1990) did not record beyond 24 hours and thresholds to noxious thermal stimulation did

not return to normal. Moreover, in the latter study the standard errors of the mean were greater than 1 at every time point after the administration of carprofen, limiting the conclusions which may be drawn from the work.

The antinociceptive properties of carprofen have been demonstrated in inflammatory models *e. g.* phenylquinone-induced mouse writhing and rat yeast paw tests (Randall and Baruth, 1976; Strub *et al.* , 1982), while the sensitivity of non-inflamed tissue was not affected. The present study in sheep confirmed these findings. It is interesting to note that carprofen is a relatively weak inhibitor of cyclooxygenase and that effective anti-inflammatory and analgesic doses of carprofen in laboratory mammals do not influence the production of cyclooxygenase (Strub *et al.* , 1982; Baruth *et al*, 1985), suggesting that other mechanisms may play a role in the antinociceptive properties of the drug. Although these mechanisms have not been elucidated, it appears that carprofen exerts antinociceptive effects only in the presence of an inflammatory focus. There is no evidence to date that carprofen acts to inhibit lipoxygenase, although it has been shown to be a weak inhibitor of phospholipase A_2, one of the enzymes involved in the release of arachidonic acid from membrane bound sites (Hope and Welton, 1983). Inhibition of release of arachidonic acid would influence production, not only of the cyclic endoperoxides such as PGG_2, but also the formation of the primary prostanoids *i. e.* PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2. Moreover, substrate for lipoxygenase metabolism also would be diminished, limiting production of the leukotrienes. Although laboratory models using pain induced by an inflammatory process have shown carprofen to be antinociceptive (Strub *et al.* , 1982; Baruth *et al*, 1985), such models would not distinguish between possible modes of action *i. e.* inhibition of cyclooxygenase or inhibition of phospholipase.

Inspection of thresholds to noxious thermal stimulation after administration of flunixin, 1.0 and 2.0 mg/kg, IV, indicated an elevation in thresholds, which persisted for a period of approximately 2 hours. Schatzmann *et al.* (1990) did not detect an elevation in cutaneous thresholds to noxious thermal stimulation after administration of flunixin, 1.1 mg/kg, IV, which supported previous findings using similar models in horses at a higher dose rate, 2.2 mg/kg, IM (Lumb *et al.* , 1983; Kalpravidh *et al.* , 1984). The analgesic efficacy of flunixin, 2.2 mg/kg, IM, against experimentally induced visceral pain also was examined in the latter 2 studies, and it was reported that although flunixin is an effective analgesic for clinical visceral pain, it was ineffective in these models (Vernimb and Hennessey, 1977). However, recently it has been reported that flunixin, 2.2 mg/kg, IV, in normal sheep caused a significant rise in thresholds to noxious mechanical stimulation, for a period of at least 1 hour, with a maximal effect at 30 minutes after administration (Chambers *et al.* , 1993b). Pre-treatment with the opioid antagonist, naloxone, prevented the hypoalgesic response and the authors suggested that flunixin may be acting *via* a descending system which includes an

opioidergic link. In the present study, thresholds to noxious thermal and mechanical stimulation were recorded every 30 minutes and it is possible that an increase in mechanical thresholds may have been obscured between sampling points. However, subsequent investigations in normal sheep with more frequent readings taken over 60 minutes (data not shown), also failed to detect the hypoalgesic effect of flunixin in normal sheep demonstrated by Chambers *et al.* (1993b). The antinociceptive properties of flunixin were initially evaluated using the standard battery of tests for NSAIDs (see chapter 1 and 4), including the acetic acid-induced mouse writhing and rat yeast paw tests (Ciafalo *et al.* , 1975), and it was shown that it was either equipotent, or more potent, than pentazocine, a partial opioid agonist, and pethidine or codeine, full opioid agonists. Interestingly, Ciofalo *et al.* (1977) also demonstrated that flunixin elevated the tail shock thresholds in normal monkeys, and commented that this was an unusual feature for a drug thought to exert its antinociceptive effects by inhibiting the enzyme, cyclooxygenase. In contrast, flunixin was not effective at elevating the threshold to heat-evoked tail flick in mice (Ciofalo *et al.* , 1977). The elevation in thresholds to noxious stimulation reported in normal animals after administration of flunixin (Ciofalo *et al.* , 1977; Chambers *et al.* , 1993b), suggests that this drug may not act by inhibiting cyclooxygenase alone, and as proposed by the latter authors, a second, possibly central mode of action may be of importance. However, it is apparent that the antinociceptive effects of flunixin against physiological pain are very variable, and are not detected in every test system.

Inhibition of serum thromboxane production may be used as an indicator of serum cyclooxygenase inhibition (Vane, 1971). Flunixin, 1.1 and 2.2 mg/kg, IM, in cattle caused a 94 and 99 % inhibition of serum thromboxane B_2 production (TXB_2) respectively, 6 hours after drug administration (Lees *et al.* , 1991a). Similarly, production of PGE_2 in inflammatory exudate in cattle was inhibited by 94 and 95 % respectively. Serum levels of flunixin of approximately 2 μg/ml have been shown to result in >90 % inhibition of serum TXB_2 in dogs (McKellar *et al.* , 1989), while values below this level did not cause significant inhibition of serum TXB_2 in horses and cats (Lees and Taylor, 1991; Soma *et al.* , 1992). Similar studies have not been conducted in sheep, although it is considered likely that doses used in the present studies would be sufficient to cause significant inhibition of cyclooxygenase *in vivo*. However, if the antinociceptive activity of flunixin is related to the degree of inhibition of cyclooxygenase, it is important that such studies are carried out. However, levels of TXB_2 in sheep are low (McKellar *et al.* , 1990a) and accurate determination of inhibition would be difficult.

The results of the present study indicate that the prostaglandins are probably not involved in mediating nociceptive information processing in physiological pain in sheep.

3.4.2 Study 2

Thresholds to noxious mechanical stimulation in farm sheep which were not familiar with mechanical threshold testing were found to be significantly different from those recorded in experimental sheep, familiar with the test system. This occurred despite a 'settling in' period in the test area after sheep had been collected from the field, or shed in which they were maintained. Moreover, although sheep were penned individually during the period of threshold testing, they were not separated visually from their usual flock mates, which were held in an adjacent pen. These measures have been recommended to limit stress in experimental sheep (Livingston *et al.* , 1992a). However, exposure of the 'naive' farm sheep to the testing procedure over a period of 3 days caused a significant change in the thresholds to noxious mechanical stimulation, and by day 3, the thresholds were not significantly different from those recorded in experimental sheep (study 1). Thus it appears that 'naive' sheep become accustomed to the testing procedure over a period of 3 days, a finding supported by studies in laboratory rats (Taiwo *et al.* , 1989). The latter workers studied thresholds in 2 groups of rats over a 4 day period using a mechanical nociceptive paw withdrawal reflex, before and after administration of a hyperalgesic agent, bradykinin, or saline. They reported that the baseline thresholds recorded on the fourth day of testing were significantly lower than those recorded on the first day of the study. There was no significant difference between thresholds recorded on the third and fourth day of testing. Moreover, they demonstrated that the unexpected hyperalgesia shown after intraplantar injection of saline on day 1, was no longer demonstrable after training. This differed from the findings after administration of bradykinin, which always caused hyperalgesia after injection. Taiwo *et al.* (1989) suggested that the increase in sensitivity achieved after a training period in rats may be crucial where the expected changes in threshold after drug treatment are small, and the work presented here supports these findings strongly.

The farm sheep were all Border Leicester x Scottish Blackface (Greyface) and only 3 of the experimental sheep were of this breed, but derived from the same flock. Moreover, 5 of the experimental sheep were neutered males, whereas all the farm sheep were female. Preliminary studies indicated that rams had higher thresholds to noxious mechanical stimulation than females. However, this was not true of neutered males. Although differences in the extent and duration of xylazine analgesia has been reported between different breeds of sheep using mechanical threshold testing apparatus similar to that used in the present study (Ley *et al.* , 1990a), there have been no reports of differences in pre-treatment thresholds to noxious mechanical stimulation between breeds or sexes. The experimental sheep were maintained indoors at all times whereas, the farm sheep were maintained either indoors or in a field. Hair was not clipped from the antebrachium of either

group prior to positioning of the leg unit. It is possible that the differences recorded may be related to the density of the hair on the limb, with sheep maintained outdoors having more dense hair and possibly less sensitivity to the threshold testing unit. However, the fact that thresholds reduced over 3 days to levels similar to those recorded in experimental sheep, refutes this. Although sheep in the present study were confined to a small pen, they were not physically restrained, and it is interesting to note that they appeared to become less anxious over the 3 day testing period when the operator entered the pen to position the leg unit of the threshold testing apparatus.

It has been suggested by Hayes *et al.* (1978) that exposure to pain can activate endogenous systems which regulate nociceptive input. However, these workers recognised that pain alone may not be the only factor, but that stress associated with noxious stimulation or restraint may play an important modulatory role. They demonstrated that although noxious stimuli produced either a narcotic or non-narcotic mediated analgesia, stress alone is not always sufficient to produce a non-narcotic analgesia. They also commented that stress-induced increases in adrenocorticotrophic hormone (ACTH) or endogenous corticosteroids were unlikely to mediate either narcotic or non-narcotic mediated stress-induced analgesia. Stress-induced increases in ACTH and cortisol have been shown in sheep (Hargreaves and Hutson, 1990a; Parrott, 1990; Apple *et al.* , 1992) although concomitant analgesia has not been described.

Various neurotransmitters have been implicated in stress-induced analgesia including 5-hydroxytryptamine, dopamine, noradrenaline and endogenous opioids *e. g.* β-endorphin, (Akil *et al.* , 1986; Curzon *et al.* , 1986; Ademola and Hart, 1990), but elucidation of the characteristics of stress-induced analgesia depend not only of the species under investigation, but the type, duration and intensity of the stimulus (Ademola and Hart, 1990) making progress in the field slow. Parrott (1990) investigated the physiological responses to isolation in sheep and demonstrated elevated levels of cortisol and noradrenaline, a transient reduction in vasopressin and very little effect on prolactin, adrenaline or dopamine. In the present study, it is important to note that sheep were not visually isolated from their flock mates, and in addition limited tactile contact between sheep was possible. Elevations in plasma concentrations of β-endorphin and β-lipotrophin during shearing and electroimmobilisation have also been reported (Jephcott *et al.* , 1987). However, Wood *et al.* , (1991) showed that pre-treatment with naloxone did not prevent significant elevations in plasma cortisol levels in lambs castrated and tail docked without anaesthesia, and they suggested that sheep may have a limited ability to reduce pain and distress by release of endogenous opioids. Recently, Rovati *et al.* (1990) demonstrated that administration of the anxiolytic benzodiazepines, diazepam and clonazepam, reduced the analgesia observed after inescapable footshock in rats. Moreover, they showed that specific benzodiazepine receptor

antagonists increased the observed analgesia whereas inverse agonists did not alter the response. Although the initial findings of these authors appear encouraging, suggesting that anxiety plays an important role in stress-induced analgesia, the other findings are difficult to explain. The inverse agonists used in the study were anxiogenic and consequently would be expected to elevate thresholds, while the antagonists ought to have no effect on the receptor. This is inconsistent with their findings. Jephcott *et al.* (1988) showed that pre-treatment with diazepam did not limit or abolish the increase in plasma cortisol seen in sheep during electroimmobilisation.

Stress-induced analgesia may have caused the elevation of thresholds to noxious mechanical stimulation found in 'naive' sheep in the present study. However, determination of plasma concentration of cortisol or other stress released agents was not carried out, and therefore, it is impossible to relate the fall in thresholds over the 3 day test period to a comparable alteration in plasma levels of these agents. Pilot studies using experimental sheep accustomed to the testing procedure have indicated that physical and visual separation from the group caused marked elevations of thresholds to noxious mechanical stimulation. Hargreaves and Hutson (1990b) demonstrated that repeated exposure of sheep to sham shearing did not change the peak cortisol response, but showed that plasma concentrations declined more rapidly to pre-handling values at the end of the training period. The training period in the latter study extended over a period of 8 weeks with sheep exposed to the stressor only once every 14 days, and so it is difficult to draw comparisons with the present study. Similarly, Jephcott *et al.* (1986) found that repeated exposure to electroimmobilisation, twice daily for 2 days, did not result in diminution of the measured cortisol, prolactin, β-endorphin or β-lipotrophin response in sheep. However, Hargreaves and Hutson (1990b) suggested that repetition may be more effective in lowering the stress response to less severe stimuli, as the sheep in their study demonstrated a strong conditioned aversive response to shearing.

Thresholds to noxious mechanical stimulation in 'naive' farm sheep suffering from footrot, were not significantly different from thresholds recorded in normal sheep from the same farm. It is interesting to note that when thresholds were evaluated over a period of 3 consecutive days in these sheep, the thresholds recorded on day 3 were not significantly different from those recorded on day 1, unlike the pattern shown in 'naive' farm controls. This may indicate that sheep suffering chronic pain have a stress-induced analgesia which persists when they are accustomed to the testing procedure. Sheep suffering chronic pain, as a result of footrot, have been shown to have significantly lower plasma concentrations of cortisol than control sheep, with increased levels of plasma prolactin, and it has been demonstrated that plasma levels of these agents return towards normal values with resolution of the condition (Ley *et al.* , 1991b). These findings obviously demonstrate that

physiological responses to chronic pain in sheep differ from those found in acute pain (*vide supra*). Presently, there are no studies which have evaluated the role of endogenous opioids in chronic pain in sheep and it is possible they may play a role.

The present study included 4 sheep with footrot present in both forefeet, and therefore sheep were required to weight bear on an affected limb in order to withdraw the contralateral limb from the noxious stimulus. This may have contributed to the high threshold values recorded over the test period. However, only 4 of the 10 sheep with unilateral lameness tested in study 2 were found to have a threshold lower than the mean threshold for control 'naive' sheep, and not different from experimental sheep. This lends weight to the finding that in fact thresholds to noxious mechanical stimulation were not reduced in sheep suffering from footrot in this study. This is contrary to the findings of Ley *et al.* (1989) who demonstrated that sheep infected with footrot for periods of not less than 1 week, had significantly lower thresholds to noxious mechanical stimulation than control sheep. Moreover, they demonstrated that local anaesthetic block of the affected foot returned thresholds to normal. In mildly affected sheep, thresholds to noxious mechanical stimulation returned to normal after treatment and resolution of the lesion, whereas in sheep which had been more severely affected, resolution of the lesion did not return thresholds to normal. Thresholds to noxious thermal stimulation were not changed.

There are a number of differences between the present study and that of Ley *et al.* (1989). Firstly, although the breed of sheep used by Ley *et al.* (1989) was not mentioned, it is likely that they were a different breed to those used in the present study. Secondly, all sheep used in the original study were suffering from footrot in only 1 fore foot, whereas <50 % of sheep used in the present study had footrot present in only 1 forefoot. Sheep used in the present study had been suffering from footrot for a period of at least 1 week, which is similar to the duration of infection reported by Ley *et al.* (1989; 1990b; 1991a). However, it was not known for how long sheep had been infected with footrot before that time in the present study, and it was not reported by Ley *et al.* (1989). Moreover, although the farm controls were not suffering from footrot at the time of the study, and for at least 1 week prior to the study, the history of individual sheep was not known. Ley *et al.* (1989) demonstrated that thresholds to noxious mechanical stimulation were reduced in sheep severely affected with footrot for a prolonged period of at least 3 weeks after resolution of the condition. Therefore, previous infection with footrot may influence control thresholds to noxious mechanical stimulation derived from apparently normal sheep. It is interesting to note that data published subsequently by these workers have not demonstrated a significant reduction in thresholds to noxious mechanical stimulation in sheep suffering from footrot (Ley *et al.*, 1990b; 1991a).

The finding that flunixin and carprofen did not demonstrate antinociceptive activity in noxious mechanical and thermal test systems in normal 'naive' sheep perhaps was not surprising (Winder, 1959). Consequently, because a reduction in threshold to noxious mechanical stimulation was not found in the present study, it was considered unlikely that administration of NSAIDs would alter the thresholds in lame sheep. Indeed, administration of flunixin, 1.0 and 2.0 mg/kg, IV, to sheep suffering from footrot did not change thresholds in the 30 minute period after administration. However, there was a significant difference between thresholds recorded pre- and post-administration for sheep between the 2 groups. Sheep were allocated to treatment groups randomly and there were no significant differences between total lesion and lameness scores.

In contrast, repeat administration of flunixin, 1.0 mg/kg, IV, over a period of 3 days did cause a reduction in mechanical thresholds to noxious stimulation. Unlike control sheep, thresholds on day 2 were significantly elevated, although subsequently, on day 3 and 4, thresholds were significantly lower than those recorded on the first day. The elevated thresholds recorded on day 2 may be linked to paring the feet of the sheep after testing on day 1. It is also possible that anticipation of an aversive procedure contributed to the development of a stress-induced analgesia (Hargreaves and Hutson, 1990a; 1990b). The reduced thresholds recorded on day 3 and 4 may have been related to the administration of flunixin meglumine. If this was the case, flunixin was exerting 1 of 2 effects. Firstly, it may have caused a hyperalgesia. This would be contrary to available evidence on the mode of action of flunixin (Lees *et al.* , 1991a), in addition to evidence from laboratory studies in which flunixin increased thresholds to noxious stimulation in hyperalgesic states (Ciofalo *et al.* , 1975; 1977), and from evidence in normal sheep where flunixin has been shown to cause hypoalgesia (Chambers *et al.* , 1993b). Secondly, flunixin may have exerted an effect such that sheep suffering from footrot responded to repeated threshold testing in a manner similar to control sheep *i. e.* over a period of 3 days, thresholds to noxious mechanical stimulation fell to a level at which they were no longer significantly different from values obtained from experimental sheep. A possible explanation for this is that the anti-inflammatory, and reported analgesic effects of flunixin (Vernimb and Hennessey, 1977; Reid and Nolan, 1991), made the sheep less reluctant to bear weight on the contralateral limb which, in most cases also was affected with footrot.

Quantification of clinical pain is difficult for several reasons. Pain is a subjective phenomenon and just as in humans, it must be assumed that individual animals will react to similar levels of pain by different degrees. However, animals cannot convey what they perceive as pain verbally and so the degree of pain must be inferred by observation. Currently, in the case of footrot, severity of pain may be assessed by examining the severity

of lameness or by grading the pathological changes that are present, although evaluation of stress hormones may prove useful (Ley *et al.* , 1991b). This approach is flawed because of the difficulty in equating physical changes with severity of pain *e. g.* personal experience has shown that sheep with grade 1 footrot may be severely lame while those with grade 4 lameness may be virtually sound. Combining the assessment of physical changes and observed lameness therefore may help to overcome this problem (Ley *et al.* , 1989). However, when clinical cases are used in the quantification of pain, the history of the sheep is not always known, and it is possible that sheep may have suffered recurring bouts of footrot which may affect their threshold response to noxious stimulation (*vide supra*).

3.4.3 Study 3

Because of the limitations of evaluating chronic clinical pain (*vide supra*), it was decided to investigate threshold responses to noxious mechanical and thermal stimulation in a group of healthy sheep undergoing identical surgical procedures. Elective surgery of this type presents an unique opportunity to limit variation within the test system *e. g.* exclusion of sheep suffering from footrot or any other infectious or non-infectious disease; similar pre-, intra- and post-anaesthetic management *etc.*

There have been conflicting reports in the human literature of changes in sensory thresholds in the post-operative period (Ekblom and Hansson, 1987; Hansson *et al.*, 1988; Lund *et al.* , 1990; Dahl *et al.* , 1992). Most recently, Dahl *et al.* (1992) demonstrated post-operative hyperalgesia in patients which has undergone abdominal surgery. In contrast to the present study, Dahl *et al.* (1992) used electrical stimulation to evaluate pain thresholds, and hyperalgesia was recorded for up to 48 hours post-operatively. While an acute surgical stimulus did not change thresholds to noxious mechanical stimulation in the present study, demonstration of a thermal hyperalgesia in the post-operative period using the ear clip method perhaps is puzzling. The sheep in the present study had a caudal ventral midline laparotomy. Therefore, it could be anticipated that a primary hyperalgesia may have existed in the area local to tissue injury (Raja *et al.* , 1988), responsive to both thermal and mechanical stimulation (Raja *et al.* , 1984). Because of the nature of the operation, lifting and stretching of the wound edges was unavoidable. This potentially could have extended the area of secondary hyperalgesia demonstrable on the ventral body surface. However, the extent of secondary hyperalgesia was not investigated in the present study. The ear is innervated primarily by the facial, trigeminal and vagal nerves which derive from the facial, trigeminal and vagal nuclei located within the brainstem (Sissons and Grossman, 1975). Innervation of the ventral abdominal wall is from nerves arising from the 8th thoracic to the 3rd lumbar spinal nerves with afferent fibres terminating in the dorsal horn of the spinal cord. Consequently, the detection of thermal hyperalgesia suggests the involvement of a

central component, not limited to the region of the spinal cord innervating the ventral abdominal wall.

Thiopentone is a barbiturate that has been reported to cause development of hyperalgesia (Clutton-Brock, 1960; Dundee, 1960), although a recent *in vitro* study demonstrated that barbiturates depressed the nociceptive-related slow ventral root potential, and enhanced the antinociceptive dorsal root potential in neonatal rat spinal cord (Jewett *et al.* , 1992). Moreover, in the previous year, an *in vivo* study in human volunteers showed an analgesic effect of thiopentone after subhypnotic doses (Anker-Moller *et al.* , 1991). Halothane is believed to exert very little analgesic effect although nitrous oxide has a recognised analgesic effect (Hall and Clarke, 1991). However, thresholds to mechanical and thermal noxious stimulation in sheep anaesthetised for a period of 20 minutes using these agents in the absence of surgery, were not significantly altered for at least 2 hours post-operatively. This indicated that in the present study neither an analgesic, nor a hyperalgesic effect of thiopentone was demonstrated. However, it should noted that the first post-operative assessment of thresholds to noxious stimulation was made 50 minutes after thiopentone administration.

The NSAIDs are being used increasingly in human surgery, either in combination with other analgesic agents, or on their own to provide relief of peri-operative pain (Milne and Twomey, 1980). There have been many clinical studies comparing both the analgesic effects of various NSAIDs and the opioid 'sparing' effects of NSAIDs when administered in combination with full or partial opioid agonists (Dahl and Kehlet, 1991). It has been demonstrated that pre- and post-operative administration of NSAIDs can reduce pain intensity, opioid requirement, or both, in humans which have undergone soft-tissue surgery (Owen *et al.* , 1986; Gillies *et al.* , 1987; Hodsman *et al.* , 1987; Dueholm *et al.* , 1989), and similar results have been demonstrated in humans after orthopaedic surgery (Breivik *et al.* , 1984; Dahl and Kehlet, 1991). It has been suggested that the NSAIDs may exert central as well as peripheral analgesic or antinociceptive effects, and a recent electrophysiological study in humans demonstrated that analgesia seen after administration of the NSAID, ketoprofen, included a central component (Willer *et al.* , 1989). It was suggested that a supraspinal mechanism was involved since the effect was not demonstrable in paraplegic patients with complete spinal transection.

Limited studies have been conducted investigating the analgesic effects of NSAIDs peri-operatively in domestic species. In dogs after orthopaedic surgery Mburu *et al.* (1988), Mbugua *et al.* (1989) and Mburu (1991) demonstrated that phenylbutazone was an effective analgesic, but did not reduce post-operative swelling to the same degree as acetylsalicylic acid and paracetamol, while indomethacin was toxic. Flunixin meglumine has been reported

to provide analgesia in horses with non-surgical colic (Vernimb and Hennessey, 1977) and recently, it has been shown to provide post-operative analgesia comparable to that achieved by papaveretum, an opioid drug, for a period of at least 6 hours after administration to dogs undergoing a variety of soft-tissue and orthopaedic surgical procedures (Reid and Nolan, 1991).

Carprofen is a propionic acid derivative (Baruth *et al.* , 1985), and the finding that carprofen induced a rise in thresholds to noxious thermal stimulation after surgical intervention in sheep is interesting. It has been noted previously, that the level of antinociception observed after administration of carprofen is greater than might be expected from the level of inhibition of cyclooxygenase achieved at therapeutic doses by this drug (Strub *et al.* , 1982; Baruth *et al*, 1985). This suggests that a further mode of action of carprofen may be important. Analgesia demonstrated after administration of ketoprofen, also a propionic acid derivative (Harris and Vavra, 1985), has been shown to involve a supraspinal mechanism (Willer *et al.* , 1989), and it is possible that other propionic acid derivatives may have similar actions. Flunixin meglumine is a potent inhibitor of cyclooxygenase in a variety of domestic species (McKellar *et al.* , 1991b), and it is of equal interest that administration of flunixin prevented a reduction in thresholds to noxious thermal stimulation in the post-operative period. These findings suggest that prostaglandins are important in the development of post-operative thermal hyperalgesia, either centrally or peripherally, but a further mode of antinociceptive action cannot be ruled out for carprofen.

Spinal cyclooxygenase inhibition has been demonstrated to block thermal hyperalgesia in rats (Malmberg and Yaksh, 1992a). Interestingly, this effect was maintained when the NSAIDs were administered after induction of hyperalgesia. In the present study, treatment was administered towards the end of surgery and therefore, after release of endogenous hyperalgesic agents and stimulation of small nociceptive afferents (Woolf, 1989). Malmberg and Yaksh (1992a) evoked a hyperalgesic response by intrathecal administration of NMDA and substance P. Substance P is an excitatory neurotransmitter thought to serve as a pain transmitter (Otsuka and Yanagisawa, 1987), and is released from primary afferents (Jessel and Iversen, 1977) and recently, a role for substance P in the transmission of thermal nociceptive input from the periphery to the central nervous system (CNS) has been demonstrated (Lecci *et al.* , 1991). The excitatory amino acids, glutamate and aspartate are endogenous ligands at the NMDA receptor, and therefore, it would appear that NSAIDs, either directly or indirectly, exert a central effect at excitatory synapses. Moreover, the NSAID, flurbiprofen, has been demonstrated to act synergistically with an inhibitor (L-N^G-nitro arginine methyl ester (L-NAME)) of the enzyme nitric oxide synthase, which results in the formation of nitric oxide (NO), to enhance the central antinociceptive effect of the inhibitor (Morgan *et al.* , 1992). This is of interest because release of NO in the CNS has

been demonstrated after NMDA challenge (Garthwaite *et al.* , 1988), and it has been proposed as an intercellular messenger. If NSAIDs act at the level of the NMDA receptor to block excitatory transmission, it is possible that they may prevent release of the intercellular messenger, NO. Moreover, the NSAIDs may also serve to modulate hyperalgesia centrally by limiting production of prostaglandins in the spinal cord, which have a known hyperalgesic action (Uda *et al.* , 1990), or indeed by preventing NMDA receptor activation of the arachidonic acid cascade (Dumuis *et al.* , 1992).

The administration of full and partial opioid agonists is standard for provision of peri-operative analgesia in both medical and veterinary surgical practice. It is recognised that pre-emptive analgesia is a more practical goal than attempting to control pain once it has been initiated. Suggesting this, McQuay *et al.* (1988) showed that 56 % of patients, who had not received pre-operative analgesia, and who underwent elective orthopaedic surgery requested analgesia with 2 hours post-operatively while, only 7 % of patients who had received opioid premedication requested supplementary analgesia in the same time period. The antinociceptive activity of buprenorphine, 6 μg/kg, IV, has been investigated in normal conscious sheep (Nolan *et al.* , 1987c). A lag period of approximately 45 minutes occurred between administration of the drug and maximal response to noxious thermal stimulation, probably due to slow receptor kinetics (Hambrook and Rance, 1976). In the present study buprenorphine, 10 μg/kg, IV, administered after the onset of surgery and before the end of anaesthesia did not cause a significant elevation in thresholds to noxious thermal or mechanical stimulation. The first measurement of thresholds to noxious thermal stimulation was made 30 minutes after termination of anaesthesia, at which time Nolan *et al.* (1987c) observed a non-significant rise in thermal thresholds in normal sheep. It is possible that a maximal response was not observed in this study because buprenorphine was administered after activation of nociceptive afferents, since it has been demonstrated that the dose of morphine, a full opioid agonist, required to prevent C-fibre-induced excitability changes from occurring in the spinal cord, is less than that required to elicit a similar response once the changes are established (Woolf and Wall, 1986b). However, in the present study buprenorphine was given after the start of surgery but before the end of anaesthesia. Buprenorphine has a bell shaped dose response curve resulting in antagonistic activity at higher dose rates (Dum and Herz, 1981), and it is unclear where the dose given in the present study lies on the dose response curve. Fentanyl, a potent short acting full opioid agonist, was shown to have a limited extent and duration of analgesia in sheep suffering chronic pain when compared to normal sheep (Ley *et al.* , 1990b). Further studies have demonstrated a similar reduction in analgesia after administration of xylazine, an α_2-adrenoreceptor agonist, to sheep in chronic pain (Ley *et al.* , 1991a). Investigations of the effect of chronic or acute pain on the analgesia observed after administration of partial opioid agonists have not been conducted, but it is possible that the presence of an acutely painful

stimulus, together with the time of drug administration, may have limited the buprenorphine analgesia observed in the present study.

The NMDA receptor appears to play a crucial role in the development of central hyperalgesia (Woolf, 1991), and therefore, it was of interest that sheep administered ketamine, a non-competitive NMDA receptor antagonist, did not develop the thermal hyperalgesia demonstrated in control surgical cases. In the present study, ketamine was administered before the onset of surgery and concurrent nociceptive afferent activation, but administration of an NMDA receptor antagonist, believed to be active at the same site as ketamine (Lodge and Johnson, 1990), has been shown to abolish existing hypersensitivity (Woolf and Thompson, 1991). However, Malmberg and Yaksh (1992a) found that NMDA receptor anatagonists did not attenuate pre-existing thermal hyperalgesia. Currently, ketamine is licensed for induction of anaesthesia in sheep and therefore demonstration of an antinociceptive effect of this agent in sheep would be invaluable, since use of unlicensed agents in food producing species is limited because of residue concerns. Perhaps not surprisingly, subanaesthetic doses of ketamine have proved analgesic in humans (Owen *et al.*, 1987; Maurset *et al.*, 1989).

The discovery that laparotomy in sheep, with gentle manipulation of the uterus causes a reduction in thresholds to noxious thermal stimulation over a period of at least 2 hours is exciting. While many other workers have demonstrated hyperalgesia experimentally following administration of hyperalgesic agents, controlled studies in clinical cases have been limited. Such studies are hindered by variation in the degree of pain or extent of pathology present prior to testing (Ley *et al.*, 1989), or variation in surgical procedure and lack of appropriate controls (McQuay *et al.*, 1988), and these factors limit interpretation of data. Duration of surgery, surgical technique and peri-operative management was identical in the sheep used in the present study, and further investigations under the same conditions in larger numbers of sheep would be invaluable to further quantify the findings which have been presented.

CHAPTER 4

THE ANTINOCICEPTIVE EFFECTS OF NSAIDS IN SHEEP: 2 EXPERIMENTAL PAIN

4.1 Introduction

4.1.1 Tourniquet-induced ischaemic effects

A tourniquet is an instrument applied around an extremity for compression of blood vessels in order to control or prevent circulation distal to the tourniquet (Blass and Moore, 1984). These workers trace the history of the tourniquet back to the Romans, but it was not until 1864 that the tourniquet was used for procedures other than amputation of a limb. In 1904, Cushing described a pneumatic tourniquet suitable to allow extremity surgery. The use of a tourniquet limits blood loss during surgery and perhaps most importantly, provides a bloodless surgical field allowing surgeons to operate quickly. Application of a tourniquet also may be used to facilitate intravenous regional anaesthesia (Weaver, 1972; Bogan and Weaver, 1978).

Application of a tourniquet is not a benign procedure (Blass and Moore, 1984; Ibrahim, 1989), and arrest of circulation must be regarded as unphysiological. In humans, occlusion of blood by application of a tourniquet causes the extremity distal to the tourniquet to become pale, peripheral pulses cannot be detected and the temperature of the soft tissues declines (Mullick, 1978). Various local and systemic biochemical and physiological changes have been observed during ischaemia in both humans and animals, including reduction in venous pH (Chin *et al.* , 1976; Scott *et al.* , 1979; Singh *et al.* , 1982); changes in venous and muscle partial pressures of oxygen and carbon dioxide (Heppenstall *et al.* , 1979; Scott *et al.* , 1979; Rorabeck, 1980; Singh *et al.* , 1982); elevation in creatinine phosphokinase, an enzyme found primarily in skeletal muscle (Chin *et al.* , 1976; Heppenstall *et al.* , 1979) and alterations in blood levels of glucose, potassium and lactic acid (Heppenstall *et al.* , 1979; Scott *et al.* , 1979; Rorabeck, 1980). Shortly after reperfusion of the ischaemic extremity, systemic and local concentration of these agents have been found to return to control levels (Blass and Moore, 1984).

Muscle and nerve damage are frequent complications after use of a tourniquet (Eckhoff, 1931; Moldaver, 1954; Mullick, 1978; Patterson and Klenerman, 1979; Rorabeck, 1980). Muscular damage has been shown to be more severe directly under the area of tourniquet compression than more distally (Patterson and Klenerman, 1979). There has been much debate about the cause of nerve damage after tourniquet application. It is not known whether the damage results from mechanical compression or from ischaemic damage (Blass and Moore, 1984), but slowing of conduction velocity of affected nerves has been shown to vary with the duration and pressure of tourniquet application (Rorabeck, 1980).

Hypertension may occur due to the presence of an inflated tourniquet during surgery (Kaufman and Walts, 1982; Abrahamsen *et al.* , 1989; Copland *et al.* , 1989). Kaufman and Walts (1982) defined hypertension as a 30 % increase in either the systolic or diastolic pressure from baseline values recorded at first incision, and showed a mean latency to the development of hypertension of approximately 1 hour. This equated closely to the latency period reported by Cole (1952) before development of tourniquet pain in human patients. Similarly, Valli and Rosenberg (1985) demonstrated a hypertensive response to inflation of a tourniquet in people under general anaesthesia, but showed that a similar response did not occur after epidural or spinal anaesthetic techniques. Copland *et al.* (1989) reported that inflation of a tourniquet in anaesthetised horses increased mean arterial blood pressure (MABP) during the period of inflation. The rise in MABP was observed with the tourniquet positioned both on the forelimb and on the hindlimb, and with the tourniquet positioned on the uppermost and the dependent limb, and was greatest with the tourniquet positioned on a dependent forelimb. Interestingly, Abrahamsen *et al.* (1989) found that an increase in MABP occurred only on the first of 2 occasions when the same horse was anaesthetised twice, and in which an inflated tourniquet was used during surgery on each occasion. In all studies deflation of the tourniquet caused a rapid decline in MABP towards baseline levels. However, hypotension was not observed in horses after deflation of the tourniquet, as previously described in humans (Valli and Rosenberg, 1985). Hyperaemia and increased blood flow in the limb observed after ischaemia in humans (Larsson and Bergstom, 1978), may be sufficient to cause such a fall in MABP.

Cole (1952) reported that inflation of a tourniquet in the presence of full spinal anaesthesia resulted in the development of pain in the ischaemic limb approximately 45 to 60 minutes after application of the tourniquet. Prior to this report, only mention of discomfort in the presence of a tourniquet had been made (Allen, 1938). Cole (1952) suggested that pain resulted from compression of nerves or from ischaemic damage to nerves, and commented that tourniquet pain could be controlled by administration of the opioid analgesic, morphine. Immediate relief from tourniquet pain can be achieved by release of the tourniquet, allowing reperfusion of the ischaemic extremity (de Jong and Cullen, 1963). These workers compared both the sensation, and time to onset of tourniquet pain in human patients under spinal anaesthesia and experimental subjects. They reported few differences between the 2 groups, and suggested firstly, that tourniquet pain was transmitted *via* unmyelinated C-fibres, and secondly, that at the time of onset of tourniquet pain, larger diameter myelinated nerve fibres (Aβ) would have reduced conduction due to the effect of compression and, consequently, would not inhibit impulses carried in the smaller unmyelinated C-fibres. Moreover, it has been

demonstrated that conduction in larger afferent fibres (Aδ and Aβ) within the compressed area ceases completely after approximately 30 minutes and therefore, it is unlikely that these fibres conduct noxious information from the periphery to the central nervous system during ischaemia (Bentley and Schlapp, 1943).

The tourniquet method, or exercise and ischaemia, has been used to evoke experimental pain in both man and laboratory animals (Hewer and Keele, 1948; Hilgard, 1978). In man, a limb is made ischaemic by application of a tourniquet and exercised until no further work can be carried out. Time to termination, or work accomplished may be measured. Similarly, the time to first reported pain (threshold time) may be recorded and the subject asked to continue until no further work is possible (tolerance time). Smith *et al.* (1966) described the 'submaximum effort tourniquet technique' which examined magnitude of pain rather than tolerance times. This technique involved removing blood from the extremity by applying an elastic bandage, then positioning a tourniquet above the elastic bandage before removing the bandage. The subject was asked to complete a defined amount of work using the ischaemic extremity, which did not cause the development of pain, and finally the ischaemic extremity was rested until pain developed. Termination using the submaximum effort tourniquet method occurred after a longer period of time (20-30 minutes) than when the original methods were used (3-4 minutes) (Hilgard, 1978). Beecher (1957) commended tourniquet-induced pain as an experimental tool because the resultant pain was not sudden fleeting pain, but developed over a period of time, and persisted during the application of the tourniquet, features that more accurately reflect most clinical pain.

The use of tourniquet-induced ischaemic pain in animals is limited because animals cannot communicate pain verbally and investigators must rely on behavioural, motor or physiological indicators to assess painful stimuli in these animals (see chapter 1). Moreover, it is difficult to impose defined levels of work on these animals. Gelgor *et al.* (1986a) have described a model of ischaemic pain in rats. Ischaemia of the tail was induced by application of an inflatable cuff to the base of the rat's tail, and the cuff inflated to a pressure of 200 mm Hg. When the rat demonstrated escape behaviour, observed as grooming vigorously, jumping forward or attempting to turn round, the cuff was deflated. These workers suggested that standardizing the duration of ischaemia would lead to ethical problems because response latencies differed between animals, and they concluded that using a defined end-point *i. e.* coordinated escape behaviour, was most suited to this technique.

The tail flick reflex is used widely in rats for the study of analgesic agents (Chapman *et al.* , 1985), and Gelgor *et al.* (1986a) have used the tail flick reflex to detect post-

ischaemic hyperalgesia in rats. After a period of ischaemia, they demonstrated a reduction in tail flick latency at temperatures above 39°C which was not apparent in rats after application of a sham tourniquet. The authors of the latter study suggested that the release of endogenous mediators such as hydrogen ions, lactate and other hyperalgesic mediators (see chapter 3) may have caused sensitization of peripheral nociceptors, or that changes in neuronal excitability may have been involved, as previously proposed by Woolf (1983). Subsequently, it was shown that post-ischaemic hyperalgesia could be abolished by the pre-treatment of rats with lysine acetylsalicylate, a non-steroidal anti-inflammatory drug (NSAID) (see chapter 1), or mepyramine maleate, an antihistamine (Gelgor *et al.*, 1986b). These findings indicated that both histamine and the prostaglandins played an important role in the development of post-ischaemic hyperalgesia. It is interesting to note that although a variety of NSAIDs did not alter the latency to coordinated escape behaviour, they prevented reperfusion hyperalgesia in rats. The authors concluded that the mechanisms of tourniquet-induced pain and those of reperfusion hyperalgesia were not the same. Recently, intracerebroventricular (ICV) micro-injections of NSAIDs have been shown to abolish the phenomenon of post-ischaemic reperfusion hyperalgesia in rats (Gelgor *et al.*, 1992a), without altering the latency to coordinated escape behaviour. This work suggested a central role for either the NSAIDs directly or prostaglandins indirectly.

Neurones, which respond to noxious thermal stimulation of the tail, have been located in the ventrobasal complex of the thalamus in the rat (Guilbaud *et al.*, 1980; Gelgor *et al.*, 1988). During post-ischaemic noxious thermal stimulation of the rat tail, a reduction in threshold temperature, and temperature at peak firing rate, has been described which parallels the behavioural changes seen both during and after ischaemia of the tail (Gelgor *et al.*, 1988). A recent study by Sher *et al.* (1992) demonstrated that administration of 2 different N-methyl-D-aspartate (NMDA) receptor antagonists inhibited the development of post-ischaemic reperfusion hyperalgesia in rats, in a similar manner to the opioid receptor agonists, morphine and pethidine, but without the motor impairment recorded after administration of morphine and pethidine. The NMDA receptor has been implicated in the development of central sensitization and the amplification, prolongation and enhancement of noxious information received from the periphery (see chapter 3), and consequently, it would appear that central sensitization may be important in the development of reperfusion hyperalgesia. However, peripheral involvement cannot be excluded. It is important to note that a positive link between tourniquet-induced pain, and post-ischaemic reperfusion hyperalgesia has not been demonstrated.

4.1.2 Laboratory models for testing NSAIDs

Laboratory techniques used for assessing the effects of drugs on nociceptive responses have been reviewed (see chapter 1). The development of such models was essential to allow potential analgesic agents to be screened to identify products which were truly analgesic, and therefore, worthy of further development. The variety of different techniques reflects the difficulty that research workers have in evaluating analgesic agents because of the difficulty in defining pain and adequate relief of pain. Consequently, there is no single model which is used for the evaluation of novel analgesic compounds and instead a variety of different models are used in combination (Franklin and Abbott, 1989). The NSAIDs are anti-inflammatory, anti-pyretic and analgesic agents and their actions appear to be primarily related to inhibition of prostaglandin synthesis (Vane, 1971). However, many of the standard laboratory tests fail to detect NSAID analgesia in normal animals (Woolfe and McDonald, 1944; Winder, 1959; Gilfoil *et al.* , 1963; Romer, 1980; Otterness and Bliven, 1985), and the presence of an inflammatory focus appears to be essential. Randall and Selitto (1957) evaluated the antinociceptive effects of various NSAIDs using a modification of a rat paw pressure test which had been described by Green and Young (1951). They recorded a reduction in the threshold to mechanical pressure after injection of a 20 % suspension of brewer's yeast, 0.1 ml, into the paw, and noted that the administration of NSAIDs reduced the observed hyperalgesia, whereas the threshold for the uninflammed paw was unchanged. Various other irritants have been described for this model including carrageenan, trypsin, kaolin, elastase, dextran, hyaluronidase, serotonin and acid phosphatase (Vinegar *et al*, 1976; Otterness and Bliven, 1985). Maximum hyperalgesia after carrageenan injection has been shown to occur 2 to 3 hours after injection (Vinegar *et al.* , 1987; Yamamoto *et al.* , 1993). Originally, test drugs were administered at the same time as subplantar injection of irritant (Randall and Selitto, 1957), but later work suggested that the test drug should be administered 2 hours after irritant injection to maximise the analgesic rather than the anti-inflammatory effects of the test drugs (Winter and Flataker, 1965).

Turpentine injected into the paw of cats was shown to induce a long lasting inflammation (Frankstein *et al.* , 1965), and a modification of the technique was described by Dubuisson and Dennis (1977) where formalin, 0.05 ml of a 5 % solution, was injected into cats and rats. The pain response which was observed was biphasic (Dubuisson and Dennis, 1977). An early, brief period of intense pain was observed within the first 5 minutes; the pain then subsided for a period and finally a tonic phase of moderate pain was observed up to 60 minutes after injection. Dubuisson and Dennis (1977) suggested that the early pain phase was a result of direct stimulation of nociceptors, whereas the

tonic pain phase occurred after release of endogenous inflammatory mediators. Non-steroidal anti-inflammatory agents and steroids do not influence the early phase of the formalin response but have been shown to modify the late phase (Hunskaar *et al.*, 1986b; Hunskaar and Hole, 1987; Shibata *et al.*, 1989). In contrast, the 'classic' centrally acting analgesic agents, such as the opioids, have been demonstrated to be analgesic in both the early and late phase of formalin-induced pain (Hunskaar *et al.*, 1986b; Hunskaar and Hole, 1987; Shibata *et al.*, 1989).

In the 1950s it was observed that intraperitoneal injection of various irritants caused marked abdominal writhing in rodents (Vander Wende and Margolin, 1956; Siegmund *et al.*, 1957). More than 40 different irritants have been described for use in this assay (Otterness and Bliven, 1985), but phenylbenzoquinone (Siegmund *et al.*, 1957), acetylcholine (Collier *et al.*, 1964), acetic acid (Koster *et al.*, 1959) and sodium chloride (Collier and Schneider, 1969) are the most frequently employed. Vander Wende and Margolin (1956) injected an iodinated contrast medium intraperitoneally, and although the writhing response was blocked by local anaesthetic agents and opioids, administration of NSAIDs did not block the response. However, later studies, using weaker irritants, were able to detect NSAID analgesia (Collier *et al.*, 1968) and the assay is used widely in the evaluation of NSAIDs (Vinegar *et al.*, 1976). The writhing assays were not specific for analgesics (Whittle, 1964; Chernov *et al.*, 1967), but it was suggested that non-analgesic agents could be detected by the presence of various side effects including sedation and motor dysfunction (Whittle, 1964). Arachidonic acid and the prostanoids were reported to cause a writhing response after intraperitoneal injection, and administration of NSAIDs only limited the response to arachidonic acid (Collier and Schneider, 1972). In addition, prostaglandin E_2 (PGE_2), and the stable product of prostacyclin hydrolysis, 6-keto-$PGF_{1\alpha}$, have been detected in the peritoneal cavity after administration of the irritant, zymosan (Doherty *et al.*, 1987). The pain response in the writhing test has not been fully evaluated, but it will certainly be multi-factorial in origin, with release of prostaglandins playing an important role in sensitizing nociceptors to the effects of other endogenous, and exogenous, algesic agents (see chapter 3).

Experimentally-induced arthritis in rats has been used widely as a model of chronic inflammatory pain (reviewed by Otterness and Bliven, 1985). Various other models including the cross-perfused spleen preparation and intra-articular injection, also have been used to investigate the analgesic effects of the NSAIDs (Guzman *et al.*, 1962; Ferreira *et al.*, 1973; Moncada *et al.*, 1975), but those mentioned above are the most frequently employed.

Models of physiological pain were shown to be incapable of detecting NSAID-induced antinociception in sheep (see chapter 3). Consequently, it was decided to assess 2 different methods of experimentally-induced allodynia or hyperalgesia in sheep and to investigate the effect of 2 non-steroidal anti-inflammatory drugs on the evoked responses. The tourniquet method was chosen as a model in which the sheep would have a degree of control over the duration of the noxious stimulus, and moreover, thresholds to noxious stimulation had not been investigated during the period of ischaemia previously. In addition, intradermal injection of carrageenan was investigated to establish whether mechanical or thermal hyperalgesia developed after injection of the irritant in sheep, in which case the response to injection of NSAID could be evaluated.

4.2 Materials and Methods

4.2.1 Animals

A total of 6 sheep were used in this series of experiments. Husbandry conditions are outlined in section 2.1. Sheep were not fasted, and were weighed immediately before the experiment. A period of at least 7 days elapsed between successive experiments.

Drugs

Four drugs were used during the course of these experiments; flunixin meglumine, carprofen, fentanyl citrate and lignocaine hydrochloride. Drugs were administered as described (see section 2.2).

4.2.2 Threshold testing apparatus

The apparatus used for testing thresholds to noxious mechanical and thermal stimulation is described in sections 2.9 and 2.10 respectively, and testing procedures are described in sections 3.2.2.1 and 3.2.2.2 respectively.

4.2.3 Tourniquet

The tourniquet is described in section 2.8. The tourniquet was positioned on the forelimb proximal to the carpus and distal to the elbow after control mechanical threshold values were obtained. The leg unit was positioned proximal to the carpus but distal to the tourniquet. The tourniquet was inflated to a pressure of 300 mm Hg for a maximum period of 30 minutes, however, the tourniquet was deflated when the sheep showed signs of aversion. Preliminary studies indicated that for approximately 5 minutes following inflation of the tourniquet, sheep flexed the limb to which it was attached at regular intervals, but continued to eat or ruminate. Aversion was defined as the point at which sheep lifted the limb, on which the tourniquet was positioned, purposefully, more than 3 times in a 15 second period. The tourniquet was deflated at aversion.

4.2.4 Carrageenan

Twenty-four hours before the experiment, the site of intradermal injection was clipped. The preparation of the carrageenan solution is described in section 2.5, and the technique for intradermal (ID) injection described in section 2.6. Carrageenan or saline, 100 µl, was injected intradermally immediately distal to the carpus on the lateral aspect of the leg after control mechanical and thermal threshold values were obtained. Injections were made on alternating forelimbs on successive cross-overs. The diameter of the resulting wheal was

measured on each occasion, 120 minutes after injection of carrageenan or saline, using electronic digital calipers (Max-Cal, CP Instruments, Bishop Storford, U. K.).

4.2.5 Study design

4.2.5.1 Study 1

Five experimental sheep (sheep numbers 1-5) were used to assess behavioural effects of positioning the leg unit of the mechanical threshold testing apparatus in the presence of the uninflated tourniquet. In addition, changes in threshold to noxious mechanical stimulation were evaluated after the uninflated tourniquet had been in position for a period of 15 minutes, and again, 15 and 30 minutes after removal of the tourniquet. Lignocaine 2 %, 5 ml, IV, was injected distal to the tourniquet after inflation in 2 sheep in order to assess the effectiveness of the tourniquet. Thresholds to noxious mechanical stimulation were recorded at 5 minute intervals during inflation of the tourniquet and until thresholds returned to normal after deflation.

Mechanical thresholds were recorded from the ipsilateral limb during tourniquet inflation, and following tourniquet deflation for 30 minutes. A similar investigation was conducted with the leg unit attached to the contralateral limb.

Flunixin, 1.0 mg/kg, carprofen, 0.7 mg/kg, or saline (0.9 %), 5 ml, was administered IV after control thresholds to noxious mechanical stimulation had been recorded, and 1 hour before positioning and inflation of the tourniquet. Fentanyl, 5 µg/kg, IV, was administered 55 minutes after control thresholds to noxious mechanical stimulation had been recorded, and 5 minutes before positioning and inflation of the tourniquet. The drugs were administered in a cross-over design and the operator was not 'blind' to the drug administered. Mechanical thresholds were determined during the period of tourniquet inflation and until thresholds returned to normal after deflation.

Time to aversion was recorded in all investigations.

4.2.5.2 Study 2

Six experimental sheep (sheep numbers 1-6) were used in this study. The following treatments were investigated:

Group 1- saline 0.9 %, 100 µl, ID

Group 2- carrageenan 0.0625 %, 100 µl, ID/saline 0.9 %, 5 ml, IV

Group 3- carrageenan 0.0625 %, 100 µl, ID/flunixin, 2.0 mg/kg, IV

Group 4- carrageenan 0.0625 %, 100 µl, ID/carprofen, 4.0 mg/kg, IV

Treatments were administered in a cross-over design and the operator was not 'blind' to the drug administered. Thresholds were assessed on the ipsilateral side to intradermal injection. Control thresholds to noxious mechanical and thermal stimulation were determined before injection of saline or carrageenan, ID. Afterwards, thresholds were recorded at 30 minute intervals for 2 hours before injection of test drug or saline, IV, and thereafter for a further 4 hours. Twenty-three hours after injection of saline or carrageenan, ID, a series of thresholds was recorded over 60 minutes.

Ambient temperature was maintained between 8 and 12°C.

4.2.6 Statistical analyses

4.2.6.1 Study 1

The data were investigated for normality, using normal probability plots and correlation analysis. Oneway analysis of variance was used to determine variation between treatment groups. Statistical significance of differences within groups was evaluated by the Student's *t* test.

A Kruskal-Wallis test, for non-parametric data, was used to test for differences between time to aversion after drug or saline treatment. Statistical tests were carried out using a 5 % significance level.

4.2.6.2 Study 2

The data were investigated for normality, using normal probability plots and correlation analysis. Initially, oneway analysis of variance was used to assess differences between treatment groups for control thresholds and also thresholds recorded 24 hours later. Subsequently, the data was examined in 2 stages. Firstly, the response to intradermal injection was examined and secondly, the response to intravenous treatment was investigated. Statistical analysis was undertaken using a general linear model routine in each case. The experimental design was suitable for analysis using a 3 factor nested analysis of variance. This provided statistical tests for treatment groups, animal and time factors and their two-way interactions. Statistical significance of differences within groups was evaluated by the Student's *t* test.

Finally, the thermal data was analysed again after grouping the sheep according to the sequence in which they were injected with carrageenan *i. e.* 1st, 2nd and 3rd occasion. Oneway analysis of variance was used to assess differences between control thresholds to

noxious thermal and mechanical stimulation, and also to assess differences between thresholds recorded 120 minutes after injection of the irritant. Further analysis was carried out using a paired Student's *t* test. Statistical tests were carried out using a 5 % significance level.

4.3 Results

4.3.1 Study 1

The presence of the uninflated tourniquet above the leg unit was not resented by sheep and did not cause a significant change in threshold to noxious mechanical stimulation after a period of 15 minutes. After the tourniquet was removed, thresholds remained similar to control values for at least 30 minutes.

Thresholds to mechanical stimulation after injection of lignocaine, IV, distal to the tourniquet after inflation resulted in maximal elevation of thresholds (16N) in 2 sheep for the period of tourniquet inflation (30 min). Maximal thresholds were achieved in both sheep at the first recording after injection (10 min) and neither sheep demonstrated signs of aversion during the period of tourniquet inflation. Thresholds returned to control levels within 5 minutes of tourniquet deflation.

Figure 4.1 shows the change in threshold to noxious mechanical stimulation during and after a period of limb ischaemia, with the leg unit attached either to the contralateral or ipsilateral limb. Thresholds to mechanical stimulation, when the leg unit was attached to the ipsilateral limb, fell during ischaemia in all sheep examined (Table 4.1). Thresholds to noxious mechanical stimulation 15 minutes after tourniquet inflation (group mean, 1.7N) were significantly different ($p \leq 0.01$) from control thresholds (group mean, 3.1N). In addition, the last thresholds to noxious mechanical stimulation recorded before tourniquet deflation (group mean, 1.7N), were significantly lower than control values ($p \leq 0.01$). The mean time to aversion was 20.6 minutes. Thresholds returned to control levels within 5 minutes of deflating the tourniquet. In contrast, thresholds to mechanical stimulation did not fall when measurements of threshold were recorded from the contralateral limb (group mean pre-inflation, 3.6N *vs.* group mean before deflation, 3.4N) (Table 4.2), while the mean time to aversion, 19 minutes, was not significantly different from that recorded for the ipsilateral limb.

Thresholds to mechanical stimulation, when the leg unit was attached to the ipsilateral limb, fell during ischaemia in all sheep which received saline, IV (Table 4.3). Statistical analysis indicated that the last thresholds recorded before aversion were significantly

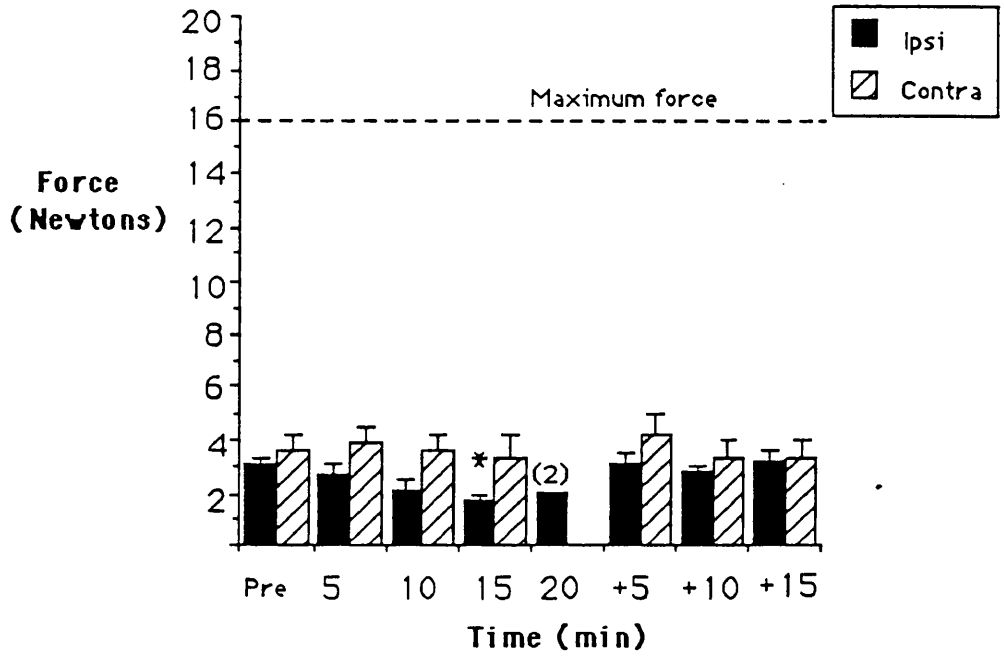

Figure 4.1. Thresholds to noxious mechanical stimulation before, during and after inflation of a pneumatic tourniquet on the forelimb of sheep.

Thresholds to noxious mechanical stimulation before (Pre), during (5 to 20 min) and after (+5 to +15 min) inflation of a pneumatic tourniquet at time 0 proximal to the leg unit of the mechanical threshold testing apparatus in a group of sheep (n=5, except when n=2 (2)) are shown. The tourniquet was positioned on either the ipsilateral (ipsi) or contralateral (contra) limb.

The response threshold (Newtons) is plotted against time (min). Control values (Pre) are the mean of at least 3 reading taken over a 30 minute period. Each point indicates the mean and standard error of the mean for recorded values at each time point.

The maximum threshold force (16 N) is indicated.

*$p \leq 0.01$ statistically different from control values.

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	Mean	±SEM
C	3.3	2.3	2.7	3.1	3.9	3.1	0.27
5	3.2	1.2	2.9	3.5	2.5	2.7	0.40
10	1.9	1.9	3.2	2.2	1.9	2.2	0.25
15	1.5	0.9	1.9	1.9	2.5	1.7*	0.26
20				2.2	1.9	2.1	
TA	20.0	19.0	18.0	24.0	22.0	20.6	1.08
+5	2.5	1.9	4.2	3.5	3.2	3.1	0.40
+15	3.2	1.9	3.2	2.9	2.9	2.8	0.24
+30	2.9	2.2	3.9	2.9	3.9	3.2	0.34

Table 4.1. Thresholds to noxious mechanical stimulation before, during and after inflation of a pneumatic tourniquet on the ipsilateral forelimb of sheep.

Individual and mean thresholds to noxious mechanical stimulation (Newtons), ± standard error of the mean (SEM), in a group of 5 sheep during and after application of an inflated tourniquet on the same limb as the threshold testing leg unit at time 0. The time to aversion (TA, min) was recorded as the tourniquet was removed and thresholds to noxious mechanical stimulation were recorded for a further 30 minutes. Control values (C) are the mean of at least 3 readings taken over a 30 minute period.

*$p \leq 0.01$ statistically different from control values.

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	Mean	±SEM
C	5.7	4.0	2.9	2.3	3.1	3.6	0.59
5	4.2	3.5	3.4	2.2	6.0	3.9	0.62
10	4.5	2.4	3.4	2.1	5.6	3.6	0.65
15	6.4	3.1	3.4	1.6	2.7	3.4	0.80
20	6.2					6.2	
TA	22.0	19.0	17.0	20.0	17.0	19.0	0.95
+5	6.5	3.2	3.5	2.2	5.6	4.2	0.80
+15	5.9	2.4	3.2	1.7	3.2	3.3	0.71
+30	5.8	2.9	3.2	2.3	2.7	3.4	0.62

Table 4.2. Thresholds to noxious mechanical stimulation before, during and after inflation of a pneumatic tourniquet on the contralateral forelimb of sheep.

Individual and mean thresholds to noxious mechanical stimulation (Newtons), ± standard error of the mean (SEM), in a group of 5 sheep during and after application of an inflated tourniquet on the contralateral limb to the threshold testing leg unit at time 0. The time to aversion (TA, min) was recorded as the tourniquet was removed and thresholds to noxious mechanical stimulation were recorded for a further 30 minutes. Control values (C) are the mean of at least 3 readings taken over a 30 minute period.

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	Mean	±SEM
C	2.8	3.0	2.8	3.3	2.1	2.8	0.20
5	1.4	1.7	3.1	2.4	1.7	2.1	0.31
10	1.3	1.9	2.6	1.6	1.4	1.8*	0.23
15	1.2	1.0	1.9	1.9	1.4	1.5**	0.18
20	1.2	1.1	2.0	1.6	1.3	1.4**	0.16
TA	24.0	23.0	21.0	22.0	23.0	22.6	0.51
+5	1.8	3.3	3.1	3.0	2.0	2.6	0.31
+15	1.8	3.0	3.0	2.7	2.1	2.5	0.24
+30	1.9	3.1	2.7	3.4	2.1	2.6	0.29
+45	1.8	3.1	3.0	3.1	2.0	2.6	0.29

Table 4.3. The effect of saline, IV, on thresholds to noxious mechanical stimulation during and after inflation of a pneumatic tourniquet on the ipsilateral forelimb of sheep.

Individual and mean thresholds to noxious mechanical stimulation (Newtons), ± standard error of the mean (SEM), in a group of 5 sheep during and after application of an inflated tourniquet on the same limb as the threshold testing leg unit at time 0. The time to aversion (TA, min) was recorded as the tourniquet was removed and thresholds to noxious mechanical stimulation were recorded for a further 45 minutes. Control values (C) are the mean of at least 3 readings taken over a 30 minute period, and were recorded before administration of saline (0.9 %), 5 ml, IV, 1 hour before the tourniquet was inflated.

* $p \leq 0.05$ / ** $p \leq 0.01$ statistically different from control values.

different from control values ($p \leq 0.05$) as described previously (*vide supra*). For all remaining treatments, fentanyl (Table 4.4), flunixin (Table 4.5) and carprofen (Table 4.6), there were no significant differences between the last thresholds recorded before aversion and control values.

Fentanyl evoked marked behavioural changes in all sheep immediately after administration. Sheep chewed frantically at ropes and bars and snatched at hay. Nystagmus was observed in some sheep, as was ataxia, while others paced back and forth within the available space. One sheep (number 3) head pressed. Thresholds to mechanical stimulation were elevated in all sheep throughout the period of ischaemia at all time points except for the final reading obtained from sheep number 3. Thresholds were significantly greater than control readings (mean, 3.4N), 5 ($p \leq 0.01$) and 10 minutes ($p \leq 0.05$) after tourniquet inflation (means, 6.6N and 5.5N, respectively) *i. e.* 10 and 15 minutes after drug administration. Final thresholds obtained during ischaemia for these sheep were higher than control values ($p \leq 0.07$), but were not significantly different from control values. Three sheep (numbers 1, 4 and 5) did not show signs of aversion before maximum cut-off time was reached. Sheep number 3 showed signs of aversion at 16 minutes. This sheep was the most agitated of all after administration of fentanyl. Thresholds in all sheep 5 minutes after deflation of the tourniquet were the same or greater than control values although these differences were not significant.

No behavioural changes were noted after administration of flunixin. Threshold to mechanical stimulation were maintained in one sheep (number 2) which did not demonstrate signs of aversion within the 30 minute test period (Table 4.5). Reduction in thresholds were observed in the remaining 4 sheep during the period of ischaemia. However, there were no significant changes between control thresholds and the final thresholds obtained during ischaemia (means, 2.8N and 1.9N, respectively).

Similarly, no behavioural changes were noted after administration of carprofen. Thresholds to mechanical stimulation were maintained in 2 of the 5 sheep investigated (numbers 1 and 2) during ischaemia (Table 4.6). Moreover, sheep numbers 1 and 2 did not demonstrate signs of aversion within the 30 minute test period. Thresholds during ischaemia in the remaining 3 sheep fell during the period of ischaemia, although sheep number 4 did not show signs of aversion before maximum cut-off time was reached. There were no significant changes between control thresholds and the final thresholds obtained during ischaemia (means, 2.6N and 3.6N, respectively). There were no significant differences between time to aversion demonstrated by the different treatment groups.

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	Mean	±SEM
C	2.7	3.0	3.7	3.2	4.4	3.4	0.30
5	4.1	6.7	5.4	7.7	9.2	6.6**	0.88
10	5.6	6.6	4.1	4.8	6.5	5.5*	0.48
15	7.8	7.5	3.1	5.4	6.7	6.1	0.86
20	4.1	8.6		6.5	4.2	5.9	0.53
25	3.6	6.2		5.8	6.1	5.4	0.61
30	4.3			5.1	5.8	5.1	0.43
TA	30.0	29.0	16.0	30.0	30.0	27.0	2.75
+5	10.8	4.0	3.7	3.6	5.2	5.5	1.36
+15	3.0	6.1	2.9	3.7	5.0	4.2	0.63
+30	2.9	3.3	3.5	3.0	3.8	3.3	0.16
+45	2.7	2.7	3.2	2.9	3.6	3.0	0.17

Table 4.4. The effect of fentanyl, IV, on thresholds to noxious mechanical stimulation during and after inflation of a pneumatic tourniquet on the ipsilateral forelimb of sheep.

Individual and mean thresholds to noxious mechanical stimulation (Newtons), ± standard error of the mean (SEM), in a group of 5 sheep during and after application of an inflated tourniquet on the same limb as the threshold testing leg unit at time 0. The time to aversion (TA, min) was recorded as the tourniquet was removed and thresholds to noxious mechanical stimulation were recorded for a further 45 minutes. Control values (C) are the mean of at least 3 readings taken over a 30 minute period, and were recorded at least 55 minutes before administration of fentanyl, 5 μg/kg, IV. Fentanyl was administered 5 minutes before the tourniquet was inflated.

* $p \leq 0.05$ / ** $p \leq 0.01$ statistically different from control values.

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	Mean	±SEM
C	3.2	2.4	2.8	3.2	2.4	2.8	0.18
5	2.0	3.5	2.7	3.5	2.5	2.8	0.29
10	1.6	4.4	2.4	2.3	1.9	2.5	0.49
15	1.5	5.6	2.9	0.7	2.0	2.5	0.84
20	1.7	4.5	2.0	0.7	1.6	2.1	0.63
25		2.8				2.8	
30		3.6				3.6	
TA	25	30	24	23	24	25.2	1.24
+5	3.6	5.0	2.9	3.0	2.7	3.4	0.41
+15	3.5	6.3	2.6	2.9	3.1	3.7	0.67
+30	3.2	6.4	2.9	2.9	3.2	3.7	0.67
+45	3.2	5.3	2.7	3.0	2.6	3.4	0.52

Table 4.5. The effect of flunixin meglumine, IV, on thresholds to noxious mechanical stimulation during and after inflation of a pneumatic tourniquet on the ipsilateral forelimb of sheep.

Individual and mean thresholds to noxious mechanical stimulation (Newtons), ± standard error of the mean (SEM), in a group of 5 sheep during and after application of an inflated tourniquet on the same limb as the threshold testing leg unit at time 0. The time to aversion (TA, min) was recorded as the tourniquet was removed and thresholds to noxious mechanical stimulation were recorded for a further 45 minutes. Control values (C) are the mean of at least 3 readings taken over a 30 minute period, and were recorded before administration of of flunixin meglumine, 1.0 mg/kg, IV, 1 hour before the tourniquet was inflated.

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	Mean	±SEM
C	2.7	2.8	2.8	2.2	2.6	2.6	0.11
5	3.0	2.0	2.1	1.4	3.1	2.3	0.32
10	2.9	2.0	2.3	1.4	2.9	2.3	0.28
15	3.3	2.9	2.4	1.5	1.5	2.3	0.36
20	3.1	3.5	2.3	1.3		2.6	0.49
25	3.1	3.5	1.5	1.6		2.4	0.46
30	3.1	3.3		1.4		2.6	0.47
TA	30	30	26	30	18	26.8	2.33
+5	3.4	2.7	2.6	2.4	2.4	2.7	0.18
+15	3.2	3.3	2.4	2.0	2.9	2.8	0.25
+30	3.3	3.2	2.9	1.4	2.7	2.7	0.34
+45	3.3	2.9	2.3	1.9	2.6	2.6	0.24

Table 4.6. The effect of carprofen, IV, on thresholds to noxious mechanical stimulation during and after inflation of a pneumatic tourniquet on the ipsilateral forelimb of sheep.

Individual and mean thresholds to noxious mechanical stimulation (Newtons), ± standard error of the mean (SEM), in a group of 5 sheep during and after application of an inflated tourniquet on the same limb as the threshold testing leg unit at time 0. The time to aversion (TA, min) was recorded as the tourniquet was removed and thresholds to noxious mechanical stimulation were recorded for a further 45 minutes. Control values (C) are the mean of at least 3 readings taken over a 30 minute period, and were recorded before administration of carprofen, 0.7 mg/kg, IV, 1 hour before the tourniquet was inflated.

4.3.2 Study 2

Intradermal injection of normal saline, 100 µl, caused a small intradermal bleb immediately after injection. This lesion was not apparent within 30 minutes of injection. Intradermal injection of carrageenan, 0.0625 %, 100 µl, also caused a small intradermal bleb immediately after injection. However, within 60 minutes of administration, a cutaneous wheal was present at the site of the original injection. The size of the wheal, approximately 1.5 to 2.0 cm x 1.5 to 2 cm, was consistent between sheep and between cross-overs. In most sheep, the wheal was not apparent 24 hours after injection, and if detected there were no distinct edges to the lesion.

Mechanical thresholds

Intradermal injection of saline did not alter thresholds to noxious mechanical stimulation over a 24 hour period (Table 4.7). However, administration of carrageenan ID, caused a significant reduction of the thresholds to noxious mechanical stimulation ($p \leq 0.01$) in group 3 (carrageenan ID, flunixin IV) (Table 4.8) compared with the other treatment groups during the first 120 minutes. Post-hoc tests did not locate the site of this significance. Statistical analysis also indicated a significant difference between sheep within treatment groups ($p \leq 0.01$). Sheep number 6 consistently responded to noxious mechanical stimulation at a greater force than the remaining sheep.

Statistical analysis of the data after administration of IV treatments (150-360 min) indicated that there was a significant difference between treatments ($p \leq 0.01$), and between sheep within treatment groups ($p \leq 0.01$). After administration of flunixin (Table 4.8), carprofen (Table 4.9) and saline (Table 4.10), IV, thresholds to noxious mechanical stimulation were significantly lower than those recorded in sheep which were administered saline ID, but received no subsequent treatment IV (Table 4.7). However, again, post-hoc tests failed to locate the site of this significance. Sheep number 6 had thresholds significantly greater, and sheep number 4 had thresholds significantly lower, than other sheep examined over the course of the study.

Thermal thresholds

Thresholds to noxious thermal stimulation after injection of saline (0.9 %), ID, were not significantly different from control thresholds for the same group of sheep over a 24 hour period (Table 4.7). However, injection of carrageenan (Table 4.8, 4.9 and 4.10), 0.0625 %, ID, significantly reduced thresholds to thermal stimulation when compared with saline (ID) controls ($p \leq 0.01$) (Fig. 4.2). Thresholds to noxious thermal stimulation were significantly lower 120 minutes after carrageenan injection in groups 2, 3 and 4 (group

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Mean	±SEM
				Thermal thresholds				
C	52.4	52.0	49.7	51.4	51.8	51.7	51.5	0.39
30	50.4	52.2	51.5	51.8	53.5	50.3	51.6	0.49
60	55.7	53.5	51.9	51.2	52.8	52.1	52.9	0.65
90	53.6	53.4	49.6	51.5	51.7	52.9	52.1	0.62
120	53.7	52.0	53.8	52.8	54.6	50.8	53.0	0.56
150	52.5	53.2	50.1	50.6	51.0	51.9	51.6	0.49
180	50.0	54.0	52.1	52.4	50.4	50.2	51.5	0.65
210	52.5	55.6	51.2	53.0	51.5	52.7	52.8	0.64
240	53.0	53.9	51.5	MD	51.7	51.0	52.2	0.53
300	51.6	51.6	51.7	53.6	51.8	51.0	51.8	0.36
360	53.4	54.2	51.9	53.8	51.8	50.9	52.7	0.54
1440	49.9	52.5	50.8	52.7	51.4	50.8	51.4	0.44
				Mechanical thresholds				
C	3.6	4.7	3.3	2.4	3.6	2.2	3.3	0.37
30	2.5	4.7	2.5	2.6	3.4	2.5	3.0	0.36
60	2.7	4.8	3.0	3.5	3.9	3.0	3.5	0.32
90	2.6	4.3	2.6	3.0	3.3	2.5	3.1	0.28
120	3.5	4.8	2.6	2.2	3.5	2.9	3.3	0.37
150	3.1	4.8	2.5	2.5	3.3	3.0	3.2	0.35
180	1.9	6.2	3.0	2.1	2.9	2.2	3.1	0.66
210	1.6	5.2	3.0	2.4	2.9	3.0	3.0	0.49
240	1.6	5.4	4.5	1.9	2.7	3.2	3.2	0.61
300	3.4	5.6	3.4	2.1	3.0	4.3	3.6	0.49
360	2.6	5.9	2.9	2.1	3.3	2.9	3.3	0.55
1440	2.6	3.4	2.9	2.4	3.5	2.9	3.0	0.18

Table 4.7. The effect of intradermal injection of saline on thresholds to noxious mechanical and thermal stimulation in sheep.

Individual and mean thresholds to noxious mechanical (Newtons) and thermal (°C) stimulation, ± standard error of the mean (SEM), in a group of 6 sheep. Control values (C) are the mean of at least 3 readings taken over a 30 minute period before injection of 100 µl saline (0.9 %) intradermally in the forelimb, at time 0.

MD= missing data

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Mean	±SEM
				Thermal thresholds				
C	49.4	54.2	50.5	51.3	51.1	52.5	51.5	0.68
30	44.6	52.6	44.4	53.9	47.2	49.5	48.7	1.64
60	48.0	55.1	44.0	47.3	50.2	47.1	48.6	1.53
90	48.0	54.3	48.6	49.6	45.4	49.6	49.3	1.19
120	46.5	52.8	46.6	50.6	45.9	47.1	48.3**	1.14
150	50.0	55.2	47.1	51.7	51.1	52.9	51.3	1.12
180	47.3	54.9	53.2	54.0	52.6	52.4	52.4	1.09
210	49.8	57.2	52.1	49.8	50.1	52.7	52.0	1.17
240	50.0	47.7	55.4	47.7	51.9	53.5	51.2	1.28
300	50.0	55.5	52.2	52.7	53.2	52.5	52.7	0.72
360	52.6	52.7	48.3	51.6	51.1	50.7	51.2	0.67
1440	51.4	52.9	51.1	53.2	50.8	51.8	51.9	0.40
				Mechanical thresholds				
C	3.9	2.5	3.5	1.5	2.9	3.4	3.0	0.35
30	2.5	2.2	2.7	1.7	1.5	3.9	2.4	0.35
60	2.5	1.6	2.7	1.7	1.2	3.9	2.3	0.40
90	2.2	2.1	3.9	1.4	2.9	3.9	2.7	0.42
120	1.7	3.2	2.5	2.1	1.9	3.4	2.5	0.29
150	3.5	2.4	3.3	2.0	3.2	2.5	2.8	0.24
180	5.5	2.7	1.9	1.5	1.7	3.1	2.7	0.61
210	5.3	2.6	1.9	2.3	2.9	3.9	3.2	0.51
240	6.5	3.0	2.5	2.0	1.6	3.1	3.1	0.72
300	5.4	3.5	1.9	2.4	4.5	3.5	3.5	0.53
360	3.4	2.4	2.0	1.4	3.0	2.9	2.5	0.30
1440	4.9	2.6	3.4	1.6	2.3	2.7	2.9	0.46

Table 4.8. The effect of intradermal injection of carrageenan on thresholds to noxious mechanical and thermal stimulation in sheep, and the response to injection of flunixin meglumine intravenously.

Individual and mean thresholds to noxious mechanical (Newtons) and thermal (°C) stimulation, ± standard error of the mean (SEM), in a group of 6 sheep. Control values (C) are the mean of at least 3 readings taken over a 30 minute period before injection of 100 µl carrageenan (0.0625 %) intradermally in the forelimb, at time 0. Flunixin meglumine, 2.0 mg/kg, was injected intravenously at 120 minutes.

**$p \leq 0.01$ statistically different from control values.

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Mean	±SEM
				Thermal thresholds				
C	51.4	52.5	53.5	53.5	52.1	51.8	52.4	0.36
30	50.6	47.6	51.2	46.6	49.7	51.6	49.6*	0.83
60	49.1	45.0	52.0	47.9	49.1	50.3	48.6*	0.96
90	44.6	48.2	51.2	47.2	49.8	48.1	48.2**	0.92
120	46.2	46.9	50.9	47.6	51.4	48.1	48.5**	0.86
150	52.4	53.3	MD	54.5	49.1	53.2	52.5	0.91
180	53.1	53.8	51.6	48.0	53.3	47.3	51.2	1.16
210	52.1	56.3	56.3	50.8	54.5	52.6	53.8	1.93
240	47.1	54.9	54.5	54.2	54.6	52.9	53.0	1.22
300	48.9	56.2	54.7	53.0	50.7	53.1	52.8	1.10
360	46.6	54.9	55.5	51.1	51.2	53.3	52.1	1.32
1440	50.3	55.5	53.4	51.8	50.9	54.0	52.7	0.81
				Mechanical thresholds				
C	3.2	3.5	3.3	2.8	4.1	3.1	3.3	0.18
30	2.3	3.3	2.9	2.3	3.3	3.5	2.9	0.22
60	3.0	2.9	3.3	2.1	2.9	2.3	2.8	0.19
90	2.2	2.3	3.7	2.5	3.1	3.1	2.8	0.24
120	3.2	3.2	2.5	2.1	3.9	2.6	2.9	0.26
150	2.9	2.2	2.4	2.2	1.6	3.4	2.5	0.26
180	3.9	2.0	3.3	1.9	3.6	2.6	2.9	0.34
210	3.3	3.2	2.3	2.0	2.3	3.1	2.7	0.23
240	4.1	3.0	2.3	2.0	2.7	3.9	3.0	0.35
300	3.0	1.7	3.1	1.9	3.1	4.3	2.9	0.39
360	2.5	2.9	3.1	2.4	2.3	2.1	2.6	0.15
1440	2.5	2.9	3.3	2.3	2.9	2.9	2.8	0.14

Table 4.9. The effect of intradermal injection of carrageenan on thresholds to noxious mechanical and thermal stimulation in sheep, and the response to injection of carprofen intravenously.

Individual and mean thresholds to noxious mechanical (Newtons) and thermal (°C) stimulation, ± standard error of the mean (SEM), in a group of 6 sheep. Control values (C) are the mean of at least 3 readings taken over a 30 minute period before injection of 100 µl carrageenan (0.0625 %) intradermally in the forelimb, at time 0. Carprofen, 4.0 mg/kg, was injected intravenously at 120 minutes.

*p≤0.05 / **p≤0.01 statistically different from control values. MD= missing data

Time (min)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Mean	±SEM
				Thermal thresholds				
C	52.9	53.0	53.5	50.6	54.6	50.0	52.4	0.72
30	46.8	53.8	53.8	46.2	51.1	49.3	50.2	1.35
60	50.1	52.3	49.1	43.4	48.7	52.2	49.3	1.33
90	47.0	50.8	48.6	45.2	50.0	48.3	48.3**	0.83
120	47.8	54.9	50.1	44.6	50.9	47.6	49.3*	1.44
150	45.1	50.2	52.3	44.9	54.8	46.4	49.0*	1.68
180	47.1	50.6	52.1	44.8	49.8	46.2	48.4**	1.15
210	48.1	54.6	51.5	44.2	52.5	50.9	50.3	1.50
240	48.3	54.5	53.6	44.6	50.1	47.3	49.7	1.55
300	48.3	54.0	50.1	46.4	49.7	51.0	49.9	1.05
360	50.1	50.1	53.5	48.5	54.1	48.3	50.8	1.01
1440	48.1	54.4	50.1	52.9	53.8	52.2	51.9	0.98
				Mechanical thresholds				
C	2.4	2.1	3.3	2.0	2.0	3.5	2.6	0.28
30	2.4	3.1	2.2	1.7	2.2	5.5	2.9	0.56
60	2.0	2.1	2.7	1.7	1.4	6.8	2.8	0.82
90	2.2	3.2	1.9	2.1	2.1	6.2	3.0	0.68
120	3.0	2.4	2.6	2.0	1.7	7.9	3.3	0.95
150	2.0	1.5	3.4	1.9	2.1	5.8	2.8	0.66
180	1.2	1.4	2.6	1.7	3.4	7.2	2.9	0.92
210	2.2	1.2	2.9	1.2	2.2	6.2	2.7	0.76
240	2.2	1.2	2.4	2.3	1.9	6.5	2.8	0.77
300	3.2	1.2	1.6	1.7	1.7	4.8	2.4	0.56
360	3.2	1.0	1.9	1.7	2.4	5.2	2.6	0.61
1440	1.9	2.5	3.1	1.8	2.3	5.5	2.9	0.56

Table 4.10. The effect of intradermal injection of carrageenan on thresholds to noxious mechanical and thermal stimulation in sheep, and the response to injection of saline, intravenously.

Individual and mean thresholds to noxious mechanical (Newtons) and thermal (°C) stimulation, ± standard error of the mean (SEM), in a group of 6 sheep. Control values (C) are the mean of at least 3 readings taken over a 30 minute period before injection of 100 µl carrageenan (0.0625 %) intradermally in the forelimb, at time 0. Saline (0.9 %), 5 ml, was injected intravenously at 120 minutes.

*$p \leq 0.05$ / **$p \leq 0.01$ statistically different from control values.

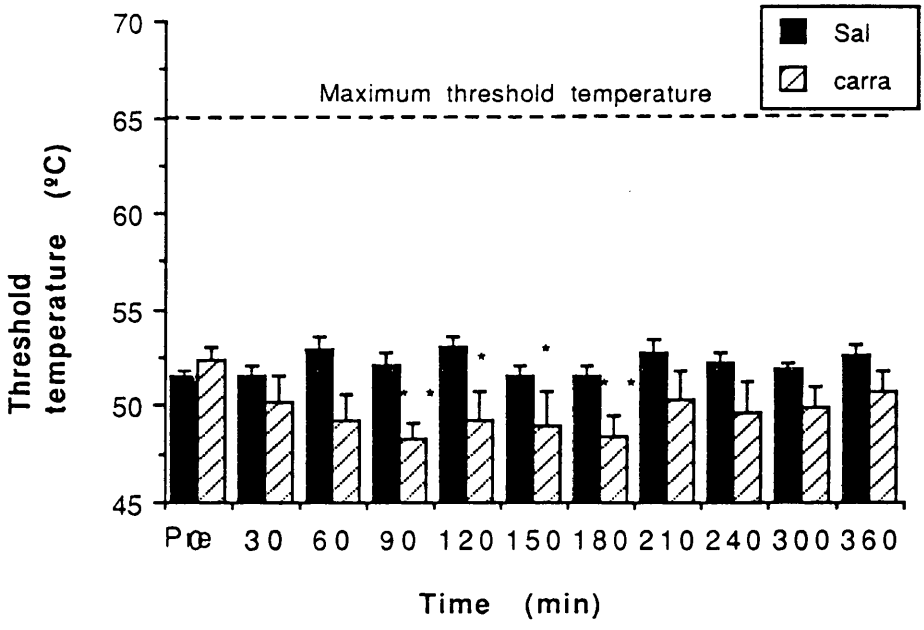

Figure 4.2. Response to injection of saline (0.9 %) (sal), 100 µl, and carrageenan (carra), 100 µl of 0.0625 %, intradermally on the lateral aspect of a forelimb in sheep.

Thresholds to noxious thermal stimulation (°C) are plotted against time (min) after intradermal injection. Each point indicates the mean and standard error of the mean for 6 sheep. The pre-test reading (pre) is the mean of at least 3 readings for each sheep over a 30 minute time period prior to intradermal injection at time 0. Sheep given carrageenan also were administered saline (0.9 %), 5 ml, intravenously, at 120 minutes.

The maximum threshold temperature (65°C) is indicated.

*$p \leq 0.05$ / **$p \leq 0.01$ statistically different from control values.

means, 49.3; 48.3; 48.5°C, respectively), than control values for those groups (group means, 52.4, 51.5, 52.4, respectively). There was a significant difference between sheep within treatment groups ($p \leq 0.01$).

Statistical analysis of the data after administration of IV treatments indicated that there was a significant difference between treatments ($p \leq 0.01$), and between sheep within treatment groups ($p \leq 0.01$). Thresholds to thermal stimulation for group 2 (carrageenan ID, saline IV) were significantly lower than thresholds recorded for the remaining 3 groups until 180 minutes.

It is interesting to note that the mean thresholds to noxious thermal stimulation after injection of carrageenan, ID, did not fall to the same extent after each occasion on which the irritant was injected (Table 4.11). There were significant differences between control thresholds to noxious thermal stimulation, and thresholds recorded 120 minutes after injection of carrageenan on the first 2 occasions only ($p \leq 0.01$) (Fig. 4.3). Unfortunately, there was not a standard period between injections of carrageenan because the study was designed as a cross-over, and not all treatments involved injection of carrageenan. Therefore, there was at least a 7 day period between successive injections of carrageenan, with a maximum period of 14 days.

	Carrageenan 1		Carrageenan 2		Carrageenan 3	
	C	120	C	120	C	120
No. 1	52.9	47.8	51.4	46.2	49.4	46.5
No. 2	52.5	46.9	54.2	52.8	53.0	54.9
No. 3	50.5	46.6	53.5	50.1	53.5	50.9
No. 4	50.6	44.6	53.5	47.6	51.3	50.6
No. 5	51.1	45.9	54.6	50.9	52.1	51.4
No. 6	52.5	47.1	50.0	47.6	51.8	48.1
Mean	51.7	46.5*	52.9	49.2*	51.9	50.4
±SEM	0.44	1.45	0.73	1.01	0.59	1.22

Table 4.11. Changes in thresholds to noxious thermal stimulation after intradermal injection of carrageenan, on 3 different occasions in sheep.

Individual and mean thresholds to noxious thermal (°C) stimulation, ± standard error of the mean (SEM), in a group of 6 sheep. Responses to injection of carrageenan, 100 µl of 0.0625 %, intradermally on the lateral aspect of a forelimb in a group of 6 sheep on 3 occasions, 7 days to 14 days, apart, are shown.

Control values (C) are the mean of at least 3 reading taken over a 30 minute period and were recorded before administration of carrageenan at time 0. Thresholds were recorded subsequently 120 minutes after injection (120).

*$p \leq 0.01$ statistically different from control values.

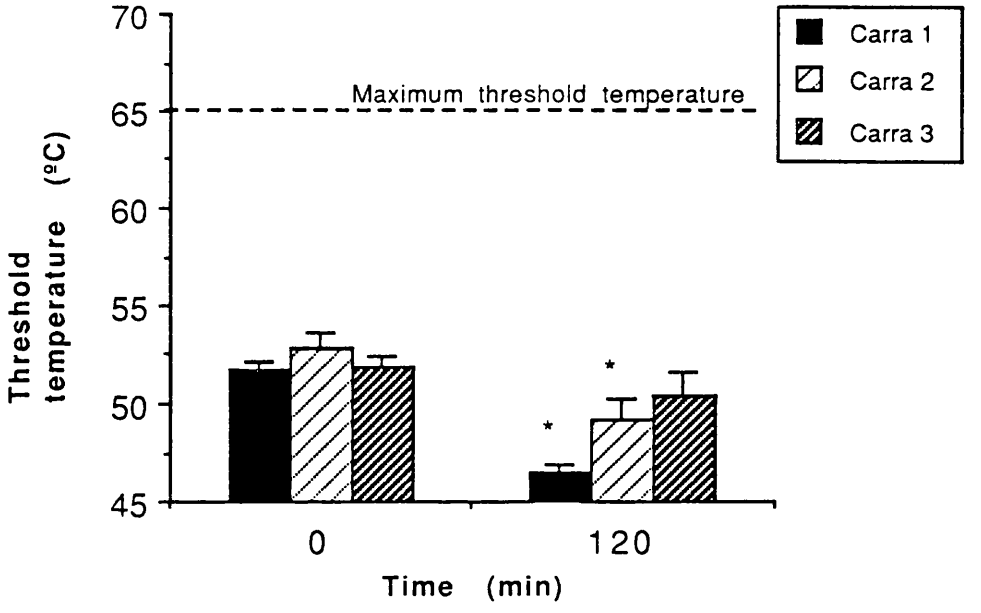

Figure 4.3. Response to injection of carrageenan (carra), 100 µl of 0.0625 %, intradermally on the lateral aspect of a forelimb in a group of 6 sheep on 3 occasions, 7 to 14 days, apart.

Thresholds to noxious thermal stimulation (°C) are plotted against time (min) after intradermal injection. Each point indicates the mean and standard error of the mean for 6 sheep. The pre-test reading (shown at time 0) is the mean of at least 3 readings for each sheep over a 30 minute time period prior to intradermal injection at time 0.

The maximum threshold temperature (65°C) is indicated.

*$p \leq 0.01$ statistically different from pre-test values.

4.4 Discussion

4.4.1. Study 1

Evaluation of tourniquet-induced pain in sheep was achieved by observing the number of times sheep lifted their leg from the ground in a purposeful manner. Therefore, it was important to demonstrate that the presence of an uninflated tourniquet did not change the normal behaviour of the sheep. Moreover, it was important to establish that the presence of the tourniquet alone did not change thresholds to noxious mechanical stimulation. Preliminary studies demonstrated that sheep did not object to the presence of the tourniquet and thresholds were not affected.

It has been recommended that surgical tourniquets should be inflated to the lowest pressure that will produce a bloodless field (Patterson and Klenerman, 1979) and pressures of approximately 100 mm Hg greater than systolic pressure are adequate for this purpose (Blass and Moore, 1984; Hagenouw *et al.* , 1986). Although, much higher pressures are occasionally employed (Hagenouw *et al.* , 1986), they do not cause greater occlusion of blood supply but may cause greater damage to the extremity (Bolton and McFarlane, 1978). In the present study, the systolic blood pressure of the sheep was not measured before each experiment and an occlusion pressure of 300 mm Hg was used in each case. This pressure was of a similar level to that used in human volunteer studies of tourniquet pain (Posner, 1984; Hagenouw *et al.* , 1986). By using local intravenous injection of lignocaine, 300 mm Hg was shown to be sufficient to occlude venous drainage of the sheep limb for a period of at least 30 minutes. However, it would be preferable to establish the systolic pressure for each sheep individually prior to tourniquet inflation, in order to limit unnecessary damage (Bolton and McFarlane, 1978).

Gelgor *et al.* (1986a) demonstrated thermal hyperalgesia, for periods of up to 90 minutes, following tourniquet-induced ischaemia of the rats tail, using a modified tail flick test, but did not record thresholds to noxious thermal stimulation during the period of tourniquet inflation (Gelgor *et al.* , 1986a; 1986b; 1992a; 1992b). In addition, hindlimb ischaemia induced by acute occlusion of the femoral artery, has been shown to act as a conditioning stimulus leading to the development of mechanical hyperalgesia in the ipsilateral rat hind paw (Sher and Mitchell, 1990). In the present study, tourniquet inflation decreased the thresholds to noxious mechanical stimulation during the period of inflation on the ipsilateral limb, but not the contralateral limb of sheep, indicating that the mechanical hyperalgesia observed was a local peripheral effect. However, reperfusion hyperalgesia was not detected, and mechanical thresholds returned to normal values immediately upon release of the tourniquet. Mechanical threshold testing apparatus similar to the equipment

used in the present study, did not detect analgesia after administration of a partial opioid agonist, buprenorphine, and a full opioid agonist, pethidine, in sheep (Nolan *et al.* , 1987c; Nolan *et al.* , 1988), that was detected by a thermal device. Similarly, post-operative hyperalgesia, not detected by mechanical threshold testing apparatus, was detected using thermal threshold testing apparatus (study 3, chapter 3). The differential sensitivity of noxious threshold testing procedures is well recognized for the opioid drugs *e. g.* pain induced by noxious thermal stimulation is modulated primarily by mu receptor agonists, whereas pain produced by chemical irritants is modulated by both mu and kappa agonists (Franklin and Abbott, 1989). Thus, while noxious mechanical stimulation detects hyperalgesia during ischaemia, it may not be sensitive enough to detect reperfusion hyperalgesia. Thresholds to noxious thermal stimulation were not recorded in the present study, however, it would be interesting to investigate if thermal hyperalgesia could be detected in the sheep model. This could be achieved either using the thermal ear clip described in chapter 2, or preferably, by a modification of the clip such that the heating resistor could be applied to the limb both during and after ischaemia.

It has been suggested that hypertension observed in anaesthetised animals that had an inflated tourniquet positioned around a limb, occurred as a result of tourniquet-induced pain (Abrahamsen *et al.* , 1989; Copland *et al.* , 1989). Consequently, it would be of interest to evaluate any change in mean arterial blood pressure which occurred during tourniquet inflation in conscious sheep. This could be achieved by either direct or indirect methods of measurement. Pilot studies in experimental sheep using an indirect measurement of blood pressure *via* a cuff positioned on the contralateral forelimb, were unsuccessful. The sheep were alarmed by the cyclic inflation and deflation of the cuff and the cuff became dislodged easily. Direct measurement of arterial blood pressure would probably provide more detailed information, and be most easily achieved in sheep in which the carotid artery had been relocated to a subcutaneous position.

Cole (1952), reporting on tourniquet pain, observed that administration of morphine before the onset of pain, frequently prevented the subsequent development of pain. Moreover, it was demonstrated recently that morphine administered intrathecally at the same time as bupivacaine resulted in fewer complaints of tourniquet pain in humans undergoing surgery of the lower extremities (Tuominen *et al.* , 1988). The authors suggested that binding of morphine to opioid receptors in the spinal cord inhibited transmission of tourniquet-induced noxious information. Fentanyl, a potent short acting μ opioid agonist, with known analgesic properties in sheep (Ley *et al.* , 1990b), elevated thresholds to noxious mechanical stimulation significantly after tourniquet inflation. Thresholds for all sheep during the period of ischaemia were greater than control values, although thresholds prior to tourniquet release were not significantly different from

control readings. The dose of fentanyl given to sheep in the present study was 3 times less than that used by Ley *et al.* (1990b), and may explain the differences in maximal threshold responses seen between the 2 studies *i. e.* in the present study the mean maximal response was almost half that reported by Ley *et al.* (1990b). Interestingly, Ley *et al.* (1990b) demonstrated that the extent and duration of fentanyl analgesia in sheep suffering chronic pain was less than that recorded in normal sheep. Thus, tourniquet pain may parallel clinical pain, as suggested by Beecher (1957), and limit the extent of fentanyl analgesia. It is possible that the motor and excitatory reactions observed in sheep after administration of fentanyl caused the increased time to aversion. This theory was proposed by Sher *et al.* (1992) to explain the increased escape latencies observed in rats after administration of pethidine and morphine, which prevented the development of reperfusion hyperalgesia. However, since it has been demonstrated that morphine can abolish C-fibre-induced excitability changes in flexor alpha-motoneurones at the level of the spinal cord (Woolf and Wall, 1986b), it is probable that μ selective opioids exert some degree of antinociceptive activity in the tourniquet model.

Pre-treatment of sheep with the NSAIDs, flunixin meglumine and carprofen, did not alter the time to aversion after inflation of the tourniquet, confirming similar findings in the rat (Gelgor *et al.* , 1986b; 1992a; 1992b). However, although thresholds to noxious mechanical stimulation fell during ischaemia in most sheep given flunixin and carprofen, overall, the reduction in recorded thresholds was not significantly different from control thresholds. This suggests an antinociceptive action of these 2 agents during tourniquet-induced pain. Pre-treatment of rats using a variety of NSAIDs, including lysine acetylsalicylate, indomethacin, ibuprofen and paracetamol has been shown to attenuate reperfusion hyperalgesia in a dose-dependent manner in the rat tail ischaemia model (Gelgor *et al.* , 1986b; 1992b). This indicated that the mechanisms of tourniquet-induced pain and reperfusion hyperalgesia differed, because administration of a NSAID did not prolong the escape latency in the same model, and administration of NSAIDs did not alter tail flick latency in the absence of ischaemia. Although the latter experiment demonstrated a well recognized phenomenon *i. e.* that generally NSAIDs do not exert antinociceptive activity in the normal animal, it does not preclude an antinociceptive action of these drugs during ischaemia when many local metabolites, including prostaglandins are released (Staszweska-Barczak *et al.* , 1976; Longhurst and Dittman, 1987). Prostaglandins released during ischaemia may act to increase activity in afferent nociceptive pathways (Staszweska-Barczak *et al.* , 1976; Longhurst and Dittman, 1987), and it has been demonstrated that peripheral Aδ and C-fibres are activated under such conditions (Chabel *et al.* , 1990; MacIver and Tanelian, 1992). Humans describe tourniquet pain as poorly localized, aching, dull and burning (de Jong and Cullen, 1963; Gelgor *et al.* , 1992b; MacIver and Tanelian, 1992) which may correspond to activation of C-fibres, and Chabel

and colleagues (1990) showed that in a rat model of tourniquet ischaemia, while spontaneous activity decreased in myelinated fibres, previously inactive C-fibres began to discharge spontaneously. Therefore, C-fibre activation may have a pivotal role in tourniquet pain. However, Gelgor *et al.* (1992b) proposed that hyperalgesia was observed during reperfusion, because it was at this stage that accumulated arachidonate was metabolized, since it was known that prostaglandin synthesis depends on a high local oxygen concentration (Lands, 1979). Results from the present study tend to contradict this theory since administration of NSAIDs attenuated the fall in thresholds to noxious mechanical stimulation during ischaemia, and also caused an increase in the time to aversion in some sheep. It is possible that these differences were noted because of the different test systems involved. However, it is important to note that the development of hyperalgesia during ischaemia has not been investigated in the rat model.

Recently NSAIDs injected intracerebroventricularly were shown to abolish reperfusion hyperalgesia in the rat tail ischaemia model (Gelgor *et al.* , 1992a), suggesting a central mode of action of the NSAIDs. This is supported by the findings of many other workers, including Malmberg and Yaksh (1992a) who demonstrated an antinociceptive effect of NSAIDs administered intrathecally in rats. Thermal hyperalgesia was evoked in the latter study by intrathecal administration of glutamate receptor agonists, including NMDA, and both pre- and post-treatment with NSAIDs attenuated the resulting hyperalgesic response. Sher *et al.* (1992) have demonstrated that systemic administration of 2 NMDA receptor antagonists, ketamine and D-2-amino-5-phosphonovalerate, inhibits reperfusion hyperalgesia. However, although Malmberg and Yaksh (1992a) showed that pre-treatment with an NMDA receptor antagonist, MK-801, attenuated the development of hyperalgesia in a non-ischaemic model, administration of the antagonist after induction of hyperalgesia, in contrast to the NSAIDs, did not. In the present study it has been demonstrated that the development of mechanical hyperalgesia during ischaemia is a local peripheral effect. However, it is possible that flunixin and carprofen may be exerting a central effect in addition to local inhibition of prostaglandin production. Therefore, it would be interesting to investigate the effects of intrathecally administered NSAIDs in sheep using the tourniquet model.

In conclusion, tourniquet-induced pain or hyperalgesia may prove to be a useful model for the investigation of the antinociceptive affects of the NSAIDs in larger domestic species. The animals have a degree of control over the duration of ischaemia, and there appears to be no residual effects after tourniquet release. The sheep in the present study were not observed to be lame afterwards. It would be valuable to establish whether thermal reperfusion hyperalgesia occurs in sheep, and if so to what extent, since this would provide a longer test period when assessing the antinociceptive or analgesic

properties of test drugs. Moreover, it may provide a model for investigation of the spinal antinociceptive effects of the NSAIDs.

4.4.2 Study 2

It was demonstrated in chapter 3 that investigation of clinical pain frequently is hampered by a variety of factors which are difficult to control or limit, such as pre-existing or concurrent disease, lack of information on the exact duration of disease or of previous episodes of the disease. Footrot in sheep has been used as a model of chronic clinical pain in sheep (Ley *et al.* , 1989) and has yielded some interesting results. However, it has proved difficult to repeat the initial findings of the latter study (study 2, chapter 3). Artificial infection of sheep with the organisms responsible for the development of footrot (see chapter 1) has been described (Egerton *et al.* , 1969), and induction of footrot in experimental sheep would allow closer control of experimental conditions. However, such experiments are difficult to justify ethically. Footrot is prevalent in the sheep flock in the United Kingdom, and presents a major welfare problem. Although, the infection responds readily to appropriate treatment, sheep can remain lame for prolonged periods of time, and sheep which have apparently recovered from infection continue to respond to noxious stimuli at a lower threshold than normal sheep for a period of weeks (Ley *et al.*, 1989).

The present study was designed to investigate the effects of intradermal injection of a mild irritant in sheep, in an attempt to mimic the inflammatory response of grade 1 footrot in this species. Carrageenan is a mucopolysaccharide extract of Irish marine algae, and is used widely in experimental models of non-immune inflammation such as the carrageenan foot oedema test, Randall-Selitto test and exudative models of inflammation. Carrageenan was selected as the irritant in the present study for several reasons. It induces a mild inflammatory reaction, of short duration (Higgins *et al.* , 1987), and it was considered important that the duration of the inflammatory response was limited for 2 reasons. Firstly, the response of the sheep to intradermal injection of an irritant was unknown, and therefore, initially it was important to ensure that any adverse effects were of limited duration. Secondly, it was considered important that if changes in thresholds to noxious stimulation were observed, that a return to control values could be documented. In addition, it has been demonstrated that carrageenan not only provides a useful animal model of acute inflammation, characterised by swelling and hyperalgesia (Winter *et al.*, 1962), but it also stimulates leukocyte migration, oedema and the formation of endogenous chemical mediators of inflammation (Otterness and Bliven, 1985; Vinegar *et al.* , 1987). Grade 1 footrot is generally limited to the interdigital space with little or no under-running of the hoof wall or sole. Where there is disruption of the epithelium, an

accumulation of exudate in the intercellular spaces and leukocyte infiltration occurs (Stewart, 1989). Therefore, it was considered that the carrageenan-evoked inflammatory response would parallel that which might be observed in the initial stages of footrot. Further support for the choice of irritant was provided by Vinegar *et al.* (1979). These workers categorised the oedema and hyperalgesia produced by a variety of irritants used experimentally according to their response to anti-inflammatory agents and serotonin antagonists, and they demonstrated that the response to carrageenan was responsive to anti-inflammatory agents and not to serotonin antagonists.

Intraplantar injection of carrageenan in rats is known to cause a reduction in the threshold to noxious mechanical stimulation using the Randall-Selitto test (Otterness and Bliven, 1985). In the present study, reduction in thresholds to noxious mechanical stimulation in sheep were not recorded after injection of saline, ID, and there was no consistent response to injection of carrageenan, ID, over a 120 minute period after injection. Only 1 of the 3 groups (Table 4.8) which received carrageenan demonstrated a significant reduction in thresholds to noxious mechanical stimulation after injection, and before drug treatment, compared to the other treatment groups. The reasons for these findings are unclear, because thresholds to noxious mechanical stimulation within group 3 were not significantly different from control values for that group. Experimental variation was kept to a minimum during the period of the study. The degree of swelling observed in the rat foot after injection of carrageenan has been shown to vary with environmental temperature (Green *et al.* , 1971), and consequently, the ambient temperature during recording was maintained between 8 and 12°C. Moreover, the carrageenan used in the present study had been prepared at least 2 weeks in advance of each experiment to improve the uniformity of wheal formation (Otterness and Bliven, 1985), and finally, all experiments were conducted at the same time of day, 0830 start time, in order to limit any circadian effects on lesional size (Labrecque *et al.* , 1981). Thus, it is unlikely that the fall in thresholds to noxious mechanical stimulation occurred as a consequence of experimental variation. Secondary hyperalgesia, whereby non-damaged tissues in the area local to the site of injury demonstrate reduced thresholds to noxious stimulation, is a well recognised phenomenon (Thalhammer and LaMotte, 1982; Raja *et al.* , 1988), and it is perhaps surprising that a definitive mechanical hyperalgesia was not recorded in the present study. However, Kocher *et al.* (1987) reported that mechanical sensitization of unmyelinated skin nociceptors in the rat did not occur after subcutaneous injection of carrageenan, although it has been shown to occur after thermal injury (Raja *et al.* , 1984). The leg unit of the mechanical threshold testing apparatus was positioned such that the blunt ended pin was pushed out against the medial aspect of the limb, and in retrospect, it would have been perhaps more appropriate, when using this model, to position the pin towards the lateral aspect of the limb because the irritant was injected laterally.

Oedema formation after intraplantar injection of carrageenan occurs in 2 phases (Vinegar *et al.* , 1968), and similarly, Vinegar *et al.* (1987) demonstrated that intraplantar injection of carrageenan in rats caused a biphasic hyperalgesic response. Within 2 minutes of injection, a short-lived hyperalgesia was recorded, which also was apparent after intraplantar saline injection. This was followed by a more prolonged period of hypoalgesia, peaking at 60 minutes post-injection. This was superceeded by a hyperalgesic phase, which reached maximum intensity by 180 minutes. It is interesting to note that from 150 to 360 minutes after administration of carrageenan or saline, ID, thresholds to noxious mechanical stimulation were lower in those sheep which had received carrageenan than in those which had received saline, ID, although the differences within groups were not significant. This suggested that a mechanical hyperalgesia was present after administration of carrageenan, ID, from 150 to 360 minutes after injection, which would correlate with the second phase of hyperalgesia demonstrated by Vinegar *et al.* (1987). The reduction in thresholds to noxious mechanical stimulation in the present study occurred despite administration of flunixin and carprofen, both NSAIDs, 2 hours after injection of carrageenan.

Hyperalgesia recorded after intraplantar administration of carrageenan may be detected using noxious thermal stimulation as well as noxious mechanical stimulation (Ren *et al.*, 1992; Yamamoto *et al.* , 1993). Intradermal administration of saline did not change thresholds to noxious thermal stimulation in sheep, but in contrast, administration of carrageenan, ID, induced hyperalgesia in all groups examined within 120 minutes of injection. Significant thermal hyperalgesia was observed after injection of saline, IV, for at least 180 minutes after intradermal injection of carrageenan. The presence of thermal hyperalgesia in this model, using an ear clip thermal device, suggests the development of central changes in response to peripheral injection of the irritant, carrageenan. In support of these findings, Ferreira *et al.* (1978a) reported that intraplantar injection of carrageenan in the rat, 120 minutes before a second intraplantar injection into the contralateral paw, reduced the time to maximal mechanical hyperalgesia in the contralateral paw, and suggested that carrageenan-evoked central changes induced this change. However, these workers did not record a decrease in response latency to noxious mechanical stimulation in the contralateral paw during the period between the 2 injections of carrageenan. In contrast, hyperalgesia after intraplantar injection of carrageenan in the rat hind paw has been demonstrated in paws distant to the injected paw, including the ipsilateral fore paw, over a similar period of time to the injected paw (Kayser and Guilbaud, 1987), and repeat intraplantar injection of saline was reported to cause a similar effect (Levine *et al.* , 1985b). In both of these studies, injection of a local anaesthetic in the inflamed area, suppressed the development of distant hyperalgesia, suggesting that afferent input from

the injured area was involved in the development of distant hyperalgesia. Both groups suggested that crossed-spinal reflexes were involved, although heterosegmental connections must be involved also to explain the expansion in neuronal receptive fields ipsilaterally (Guilbaud *et al.* , 1986). Carrageenan-induced oedema and hyperalgesia is initiated by histamine, bradykinin and serotonin, but by the third hour after injection the products of the arachidonic acid cascade play an important role (Di Rosa *et al.* , 1971; Holsapple *et al.* , 1980), and may enhance the sensitivity of cutaneous afferent fibres (Chahl and Iggo, 1977). Administration of flunixin and carprofen, IV, restored thresholds to noxious thermal stimulation back to control values within 30 minutes of drug administration, while injection of saline, IV, did not. These results suggest that the NSAIDs exerted an antinociceptive action in this model of inflammatory pain, possibly by inhibiting the development of prostaglandins peripherally. However, a central antinociceptive action of these agents is likely and cannot be excluded based on the present results.

Concentrations of 1 % carrageenan are used frequently to evoke hyperalgesia in rat paw models, using 50 to 100 µl injection volumes (Winter and Flataker, 1965; Vinegar *et al.*, 1976; Vinegar *et al.* , 1987; Wheeler-Aceto *et al.* , 1990). However, in a recent study by Yamamoto *et al.* (1993), 2 mg of carrageenan was injected into the rat paw to evoke a hyperalgesic response, approximately 32 times more than was used in the present study. While it would be of interest to repeat the control experiments in this study using higher concentrations of carrageenan, it is important to remember that the sheep cannot limit the effects of the injection of the irritant, and thus, a balance between experimental effect and limiting the pain and distress suffered by experimental animals must be made. Vinegar *et al.* (1987) commented that after subcutaneous injection of 1 % carrageenan in the rat paw, the dermis did not appear normal histologically until day 15 after injection. Moreover, after intraplantar injection of carrageenan, epidermal tissues did not return to normal until at least 21 days. A period of 14 days elapsed before intradermal injection was made on the same limb for a second time in the present study, and consequently residual tissue damage may have been evident. However, it is unlikely that carrageenan was injected into exactly the same site on the second occasion in sheep, while it would be difficult to avoid this possiblity if injecting carrageenan into the rat paw. Moreover, the concentration of carrageenan used in the present study was much lower than that shown to induced prolonged pathological changes.

Thermal hyperalgesia, which was evident 120 minutes after injection of carrageenan on the first occasion, was diminished, although still significantly reduced from control values, on the second occasion. However, thresholds to noxious thermal stimulation were not significantly reduced after injection of carrageenan on the third occasion,

suggesting tachyphylaxis. Thresholds to noxious mechanical stimulation did not show a similar trend. It is interesting to note that Ferreira *et al.* (1978a) reported a diminished oedema response after injection of carrageenan into the rat paw, 2 hours after intraplantar injection of the irritant in the contralateral paw. However, the authors did not observe a reduction in the intensity of the hyperalgesia which developed, and moreover, these findings were acute, whereas, the findings in the present study occurred over a period of weeks. The reasons for the diminished response to injection of carrageenan, ID, in sheep are not clear, however, tachyphylaxis has been observed after repeat administration of bradykinin, histamine and formylmethionyl-leucylphenylalanine, where reduced vascular permeability was observed when an intradermal site was injected on a second occasion, and compared with a new site in the same animal (Colditz, 1985). It is important to note that the alteration in response to injection of carrageenan over the study period would influence the results obtained. However, because the hyperalgesic response was diminished rather than increased after repeat administration, the observed changes do not weaken the conclusions drawn from the study.

In conclusion, these preliminary studies suggest that intradermal injection of carrageenan may be a suitable model for the investigation of the antinociceptive effects of the NSAIDs. The low concentration of carrageenan used in this study was not physically resented by the sheep, and they continued to eat and ruminate throughout the 24 hour study period. Gentle digital pressure over the site of the wheal generally caused the sheep to flex their leg in the first 6 hours after injection, but they did not flex their leg spontaneously in response to the presence of the wheal. Injection of the irritant did not affect locomotion and they were able to lie and rise without difficulty. Thus it appears that the concentration of carrageenan used in this study presents a mild stimulus, which causes a significant thermal hyperalgesia, although the mechanical response is more variable. If tachyphylaxis to carrageenan occurs, this would limit the number of studies which could be carried out on any given group of sheep. However, it is possible that a longer 'rest' period between cross-overs may resolve the problem.

The 2 models described here have provided some interesting results. Tourniquet pain, or hyperalgesia, has been demonstrated using a noxious mechanical stimulus, and appears to be a local effect, hyperalgesia during ischaemia not being evident on the contralateral limb. In contrast, the carrageenan model appears to involve a central mechanism, with thermal hyperalgesia evident at a site distant to the inflammatory stimulus.

CHAPTER 5

THE PHARMACOKINETICS OF FLUNIXIN MEGLUMINE AND CARPROFEN (RACEMATE)

5.1 Introduction

5.1.1 Chromatography

Chromatography is a process by which separation of a mixture of agents can be achieved. This process involves 2 immiscible phases, 1 of which is moving (mobile phase: gas or liquid) while the other is stationary (stationary phase: liquid or solid). Separation of the mixture is achieved by adsorption or differential partition of molecules between the mobile and the stationary phase. Thin layer chromatography (TLC), gas liquid chromatography (GLC) and column chromatography are all examples of this technique: TLC utilises a liquid mobile phase and a solid adsorbant (silica gel or alumina), applied either to a glass plate or other suitable base; GLC utilises a gaseous mobile phase and a liquid adsorbed onto an inert material; column chromatography generally uses a liquid mobile phase and a solid stationary phase. High performance (pressure) liquid chromatography (HPLC) is a refinement of the basic column chromatographic technique. Molecules within the sample may be separated by different techniques using HPLC, such as varying the mobile and stationary phases *e. g.* steric exclusion, where the molecular size restricts the diffusion of the solute through the column; adsorption, where solutes bind with differing affinities to the stationary phase; partition, where solutes dissolve in a liquid stationary phase which both coats, and is present within, a porous inert packing material; ion-exchange, where charged solutes are attracted to ionic sites bonded, or coated on a packing material.

Samples are injected onto the HPLC column *via* an injection valve. These valves comprise fixed-volume sample loops which control the volume of injection and minimise the dead space within the system. Moreover, the valve ensures the time over which the sample is injected remains constant between samples, and is independent of the viscosity of the liquid. Liquid mobile phases carry the sample through the system and must be degassed prior to use. Pre-columns are occasionally employed, and are positioned in front of the injection valve to prolong column life. Similarly, guard-columns may be positioned between the injection port and the HPLC column. These columns are packed with the same material as the main column and again act to prolong the life of the main column, in this case by removing impurities from the sample. HPLC columns are made of stainless steel or compounds such as glass, and are available in different lengths and internal diameters. The function of the HPLC column is to separate compounds within a mixture. These columns may be packed with either solid or liquid stationary phases (*vide supra*), and require high pressures to pump the mobile phase through the column. Smaller packing material particle sizes have led to the introduction of shorter HPLC columns, resulting in shorter analysis time.

The compounds which are separated are detected by various means. The output from the detector is quantifiable allowing the amount of compound in the mixture to be calculated. Various detectors can be used with HPLC. Ultra violet (uv) detectors measure the degree to which the solute absorbs uv light, while fluorescence detectors may be used to detect solutes, or derivatives of solutes, which fluoresce. The sensitivity of the latter system is greater than that achieved with the uv detector. These 2 methods, together with electrochemical detection are solute specific detectors, whereas refractive index (RI) detectors record the presence of all molecules which have a refractive index different to that of the mobile phase used in the system. The RI detector is less sensitive than the uv detector and there must be very little temperature fluctuation within the system as this can vary the refractive index of liquids.

5.1.2 Pharmacokinetics of NSAIDs

Flunixin meglumine is a potent NSAID used widely in clinical veterinary practice for both its anti-inflammatory and analgesic properties (see chapter 1). In the United Kingdom, it is currently licensed for use in the horse, dog and cow at dose rates of 1.1, 1.0 and 2.2 mg/kg respectively. Reports of the pharmacokinetics of flunixin in the ruminant have been limited to the bovine (Benitz, 1984; Hardee *et al.*, 1985; Anderson *et al.*, 1990), although they also have been investigated in the dog (Hardie *et al.*, 1985b), cat (Lees and Taylor, 1991) and horse (Chay *et al.* , 1982; Soma *et al.* , 1988).

Carprofen (±-6-chloro-α-methyl carbazole-2-acetic acid) is a novel NSAID (see chapter 1) presented in solution as a racemic mixture. In the United Kingdom, it is currently licensed for use in the horse and dog at dose rates of 0.7 and 4.0 mg/kg respectively. In the domestic species, descriptions of the pharmacokinetics of the racemate have been limited to the dog (McKellar *et al.* , 1990b), horse (McKellar *et al.* , 1991c) and lactating cow (Ludwig *et al.* , 1989) and recently, Lees *et al.* (1991c) described the pharmacokinetics of carprofen enantiomers in the horse. They showed that there were marked differences in the pharmacokinetic disposition of the individual enantiomers, although the reasons for this were not established. At all time points after administration of carprofen, the R(-)-isomer predominated and 24 hours after injection the ratio of R(-) to S(+) was approximatley 9:1. In the same year, Graser *et al.* (1991) reported a similar disposition of carprofen enantiomers, not only in horses, but also in cattle. However, in contrast to horses, the R(-):S(+) ratio showed no time dependent change over 24 hours in cattle. Limited *in vitro* investigations by the same authors indicated that incubation of the S(+)- and R(-)- isomers of carprofen in cattle and horse liver homogenates did not result in chiral inversion of either the eutomer or the distomer.

Pharmacokinetic studies are necessary to identify differences in absorption and disposition processes that may contribute to species variation in drug response. This is of particular importance where drugs are known to have a narrow therapeutic index. The NSAIDs have known toxic effects in the domestic species (Snow and Bogan, 1979; Mathews *et al.*, 1990; McKellar *et al.*, 1991b; McNeil, 1992) and consequently, it was of interest to investigate the pharmacokinetics of both flunixin meglumine and carprofen when administered IV to sheep.

5.2 Materials and Methods

5.2.1 Animals

A total of 9 Suffolk and Suffolk-cross rams and wethers, weighing 52 to 87 kg and aged 1 to 4 years were used in this study. All were free from clinical signs of systemic disease at the time of the study. Sheep numbers 1 to 5 were maintained indoors as a group. Sheep numbers 6 to 9 were maintained outdoors as part of a larger group. The entire group were brought to a holding area prior to the onset of the experiment to allow the sheep to settle. Hay and water were available *ad libitum* throughout the trial period.

Drugs

Flunixin meglumine, 1.0 mg/kg, as a 5 % solution, was injected IV in sheep numbers 1 to 9, and after an interval of not less than 14 days, the drug was administered IV to sheep numbers 1 to 5, at a dose rate of 2.0 mg/kg.

Carprofen, 0.7 mg/kg, as a 5 % mixed micelle solution, was injected IV in sheep numbers 1 to 5, and after an interval of not less than 14 days, the drug was administered IV to the same sheep, at a dose rate of 4.0 mg/kg

The test drug (flunixin, 1.0 or 2.0 mg/kg and carprofen, 0.7 or 4.0 mg/kg) was injected into a jugular vein as a bolus. Venous blood samples were collected serially from the contralateral jugular vein in the manner previously described (see section 2.4). Control samples were collected from all sheep in the period prior to injection of the test drug. Blood samples were collected at 0.03, 0.08, 0.17, 0.25, 0.33, 0.50, 0.67, 0.75, 1, 2, 4, 6, 8, 23, 24 and 32 hours after administration of flunixin, while samples after carprofen injection were collected at 0.08, 0.50, 1, 2, 4, 6, 8, 12, 24, 32, 48, 56 and 72 hours.

5.2.2 Assay for flunixin meglumine in plasma

The assay method used to determine the concentration of flunixin in plasma was the method described by McKellar *et al.* (1989).

Extraction procedure

Plasma samples were defrosted at room temperature on the day of analysis. To extract flunixin from plasma, a 2 ml sample of plasma was placed in a 60 ml conical glass-stoppered tube, and 0.6 ml of citrate phosphate buffer (pH 5) and 20 ml of diethylether were added. The stoppered tubes were shaken on a slow rotating mixer for 10 minutes

and then 15 ml of the supernatant ether was decanted into a clean 60 ml glass test tube and a further 20 ml of ether added to the tube. The stoppered tube was shaken for a further 10 minutes and then 20 ml of the supernatant ether removed and placed in the second test tube as before. Using a heating block, the 35 ml of organic solvent was evaporated under air at 50°C until approximately 7 ml remained. The remaining solvent was decanted into 10 ml conical glass tubes and the 60 ml tube was washed down with 1 ml of ether, 3 times, and the ether collected into the 10 ml tube. The remaining organic solvent was evaporated to dryness under air at 50°C. The tube was washed down with 1 ml of ether and evaporated to dryness once more. Prior to injection into an HPLC system (*vide infra*), samples were reconstituted in at least 150 µl methanol.

Quantification procedure

Stock solutions of flunixin meglumine in methanol (10, 20 and 100 µg/ml) were prepared from pure compound. These solutions were used to 'spike' blank plasma samples (2 ml) at different concentration levels, 0, 0.1, 0.5, 1.0, 2.0 and 5.0 µg/ml. These were included in each assay in order to calculate mean recovery. The expected recovery of flunixin extracted in ether was 95 % (75 % in the first 15 ml of supernatant and 20 % in the second 20 ml harvest of supernatant). The remaining 5 % of drug was discarded with the plasma and remaining ether. A standard injection (2 µg/ml) was run after every 2 sample injections in order to assess detector variability.

Chromatograph details

The mobile phase was an 80:20 mix of methanol : water containing 50 µl of a 1 in 55 dilution of a 70 % solution of perchloric acid. Prior to use, the solvent was degassed using a sonic bath and vacuum pump (Millipore U. K. , Watford, U. K.). A solvent delivery pump and manometric module (Gilson Model 302 and 802, Scotlab, Coatbridge, U. K.) were used to pump the mobile phase at 1.1 ml per minute through a Shandon column, 100 mm x 5 mm (Shandon Southern, Runcorn, U. K.), with 5 µM octadecylsilica Hypersil packing and a variable wavelength (λ) ultraviolet detector (Spectra Physics Model SP8450, Burke Electronics, Glasgow, U. K.). The uv λ was set at 287 nm and the absorbance was set at 0.005 aufs. The samples, or standards, were injected using a glass syringe into an injection port with a valve attached (Negretti and Zambra Aviation Ltd. , Southampton, U. K.). The valve had a 20 µl loop and the injection volume was 15 µl. The output from the uv detector was connected to a variable speed chart recorder (Vitatron, M. S. E. Scientific Instruments) set at a speed of 5 mm per minute.

The limit of detection achieved using this method was 10 ng/ml or better. This was based on twice background noise on the chromatogram.

Measurement of the chromatogram

The concentration of flunixin in ovine plasma was calculated from the peak height of the chromatogram. The ratio of test drug peak height and peak height of the closest standard injection, was multiplied by the concentration of the standard injection (2 µg/ml). This figure was adjusted for volume, expected drug recovery and finally multiplied by a conversion factor calculated from expected recovery from spiked samples and actual recovery of spiked samples on the day of injection.

5.2.3 Assay of carprofen racemate in plasma

Carprofen racemate was extracted from the plasma using a method developed from that described by Iwakawa *et al.* (1989).

Extraction procedure

Plasma samples were defrosted at room temperature on the day of the analysis. To extract carprofen racemate from plasma, a 0.25 ml sample of plasma was placed in a 10 ml conical glass-stoppered tube along with 62.5 µl of citrate phosphate buffer (pH 3) and 6 ml of ethyl acetate. The stoppered tube was shaken on a slow rotating mixer for 10 minutes and then 4 ml of the supernatant ethyl acetate decanted into a 10 ml glass tube. A further 4 ml of ethyl acetate was added to the residue of the first tube and the procedure repeated. Using a heating block, the 8 ml of organic solvent was evaporated under nitrogen gas (BOC Medical Gases) at 50°C. When the organic layer had completely dried, the tube was washed down using approximately 1 ml of ethyl acetate, and again evaporated to dryness under nitrogen gas at 50°C. Prior to injection into an HPLC system, the sample was reconstituted in at least 150 µl of methanol.

Quantification procedure

Stock solutions of carprofen racemate in methanol (5, 10, 20, 40, 100 µg/ml) were prepared from the racemic compound, and were used to 'spike' blank plasma samples (0.25 ml) as previously described, to give final concentrations of 0.25, 0.5, 1.0, 2.0 and 5.0 µg/ml. These standards were included in each assay in order to calculate mean recovery. The expected recovery of carprofen extracted in ethyl acetate was 88.67 % (66.67 % in the first 4 ml of supernatant and 22 % in the second 4 ml harvest of supernatant). The remaining drug was discarded with the plasma and remaining ethyl

acetate. A standard injection (5 μg/ml) was run after every 2 sample injections in order to assess detector variability.

Chromatograph details

The mobile phase was a 75:25 mix of methanol : water containing 50 μl of a 1 in 55 dilution of a 70 % solution of perchloric acid. The solvent was prepared by degassing, and delivered as described above through an identical HPLC system. The uv λ was set at 254 nm and the absorbance was set at 0.02 aufs.

The limit of detection was based on twice background noise on the chromatogram and was 100 ng/ml or better using this method.

Measurement of the chromatogram

The recovery of racemic carprofen from ovine plasma was calculated from the peak height of the chromatogram using the same system as described for flunixin meglumine (*vide supra*).

5.2.4 Pharmacokinetic analysis

The decline in plasma concentration of flunixin and carprofen with time following a single bolus IV injection were analysed for each animal using a nonlinear regression Fortran IV curve stripping computer programme, CSTRIP (Sedman and Wagner, 1976). Akaike's information criterion (AIC) (Yamaoka *et al.* , 1978) was used to confirm the number of exponents best describing each data set.

The CSTRIP program is based on standard equations describing bi- and tri-exponential decay (Baggot, 1977).

Bi-exponential- $C^t_p = Ae^{-\alpha t} + Be^{-\beta t}$

Tri-exponential- $C^t_p = Pe^{-\pi t} + Ae^{-\alpha t} + Be^{-\beta t}$

Where C^t_p is the plasma concentration of drug at time t.

P, A and B are the zero-time plasma drug concentration intercepts of the biphasic or triphasic disposition curve *i. e.* the maximum drug concentration for each exponential.

π, α and β are the rate constants for a given exponential process.

The time taken (in hours or minutes) for the plasma concentration of drug to fall by 50 %, is constant at any part of the curve and is referred to as the half-life of the drug. It may be estimated as $t_{1/2}$ = 0.693/rate constant.

Other terms used in the description of the pharmacokinetic analysis are defined as follows:

$t_{1/2\pi}$ or $t_{1/2\infty}$- distribution half-lives (min or hr).

$t_{1/2\beta}$- elimination half-life (min or hr).

$Vd_{(area)}$- apparent volume of drug distribution (ml/kg).

Dose/AUC.ß

Vc- volume of the central compartment (ml/kg).

Dose/C^0_p

$Vd_{(ss)}$- apparent volume of distribution at steady state (ml/kg).

AUMC/AUC^2

Cl_b- body clearance rate (ml/kg.min).

Dose/AUC

Kel- first-order elimination rate constant.

calculated from the ratio of Cl_b to Vc

The area under the plasma concentration-time curve for observed values ($AUC_{(obs)}$) (µg.hr/ml), or zero moment curve, was calculated using the trapezoidal rule. The area under the first moment curve ($AUMC_{(obs)}$) (µg.hr^2/ml), or the plasma concentration-time versus time curve for observed values was calculated using the equation $AUMC = P/(\pi)^2 + A/(\alpha)^2 + B/(\beta)^2$. The ratio of AUMC to AUC for any drug is the mean residence time (MRT) (hr or min). The MRT is the average time that a drug will persist in the body after administration, and the time it takes for 63.2 % of the administered dose to be eliminated from the body (Rowland and Tozer, 1989).

5.2.5 Statistical analyses

Results are quoted as mean values ± standard error of the mean (SEM). Values for half-lives are given as the harmonic mean. Derived kinetic parameters were tested with a Mann-Whitney U Test. Interdose comparisons within a group were made and statistical tests were carried out using a 5 % significance level.

5.3 Results

5.3.1 Assay of flunixin meglumine in plasma

Figure 5.1 illustrates a typical chromatograph of a plasma sample collected after administration of flunixin 1.0 mg/kg, IV. No coextracted endogenous compounds from ovine plasma interfered with the flunixin peak, nor was there evidence that any metabolites of flunixin were detected in this system.

The precision of the extraction procedure was moderate as can be seen from the between-day coefficients of variation (Table 5.1). The mean between-day coefficient of variation was 17.6 %. No flunixin meglumine was detected in the drug free plasma.

The mean plasma concentration of flunixin *vs* time following injection of 1.0 mg/kg of drug, IV, to 9 sheep is shown in Fig. 5.2. The mean plasma levels showed an initial rapid distribution phase (π phase), followed by a slower second distribution phase (α phase) and a slow elimination phase (β phase). Plots of the plasma flunixin decay curve for each individual sheep were best fitted to a tri-exponential decline and this was confirmed by applying the AIC. The individual values for the rate constants are shown in Table 5.2. The pharmacokinetic parameters calculated from the tri-exponential equations used to describe the plasma concentration *vs* time data for individual sheep are shown in Table 5.3. These indicate an initial rapid distribution half life ($t_{1/2\pi}$) of 2.3 minutes, followed by a slower second distribution phase ($t_{1/2\alpha}$) of 101 minutes. The mean elimination half-life ($t_{1/2\beta}$) was 221.7 minutes. Sheep number 2 had slow initial and second distribution phases and a slow elimination half-life, 16.1, 222.5 and 728.7 minutes, respectively.

The mean apparent volume of distribution, $Vd_{(arca)}$, was small, 248.6 ± 57.3 ml/kg, although a higher value was calculated for sheep number 2. Similarly, the mean apparent volume of distribution at steady state, $Vd_{(ss)}$, and the mean apparent volume of the central compartment, Vc were small, 166.2 ± 37.23 ml/kg and 36.3 ± 4.9 ml/kg respectively.

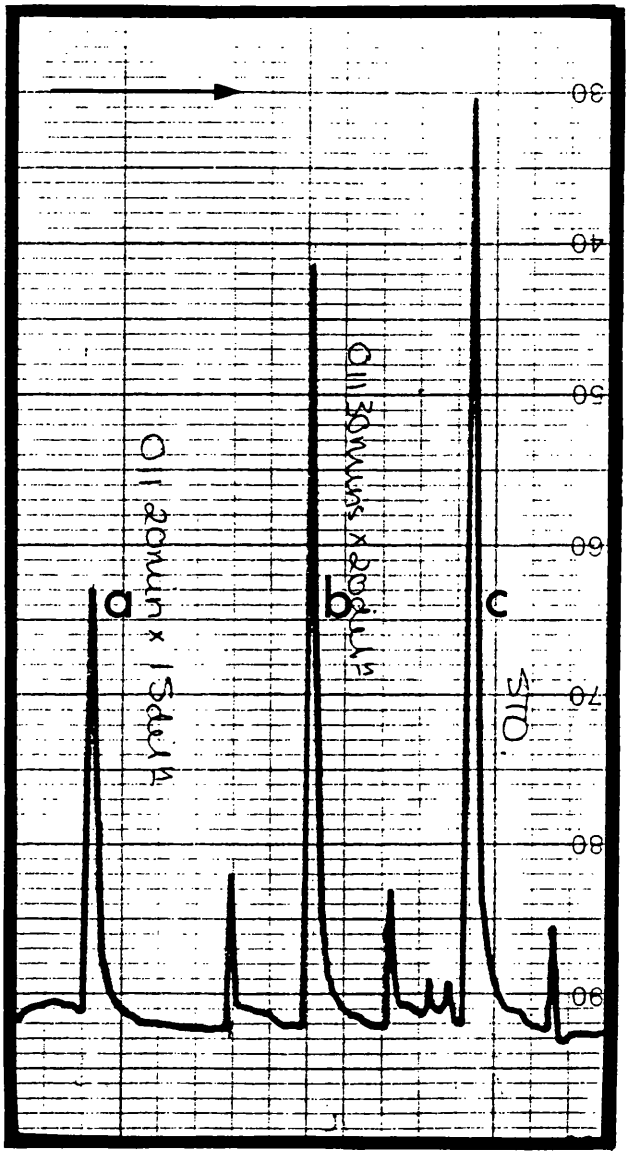

Figure 5.1. Typical chromatogram of flunixin meglumine in ovine plasma.

Key- **a** and **b**- unknown ovine plasma samples

c- 2 µg/ml flunixin meglumine in ovine plasma (standard injection)

→- direction of flow

Concentration (μg/ml)	Mean recovery (%)	Standard deviation	Coefficient of Variation (%)
0.1	82.1	17.3	21.1
0.5	91.7	14.7	16.1
1.0	97.0	16.8	17.2
2.0	89.3	19.0	21.2
5.0	94.0	11.6	12.4
Mean	90.7	15.9	17.6

Table 5.1. Mean recovery and coefficient of variation (%) of flunixin meglumine in ovine plasma (n=14).

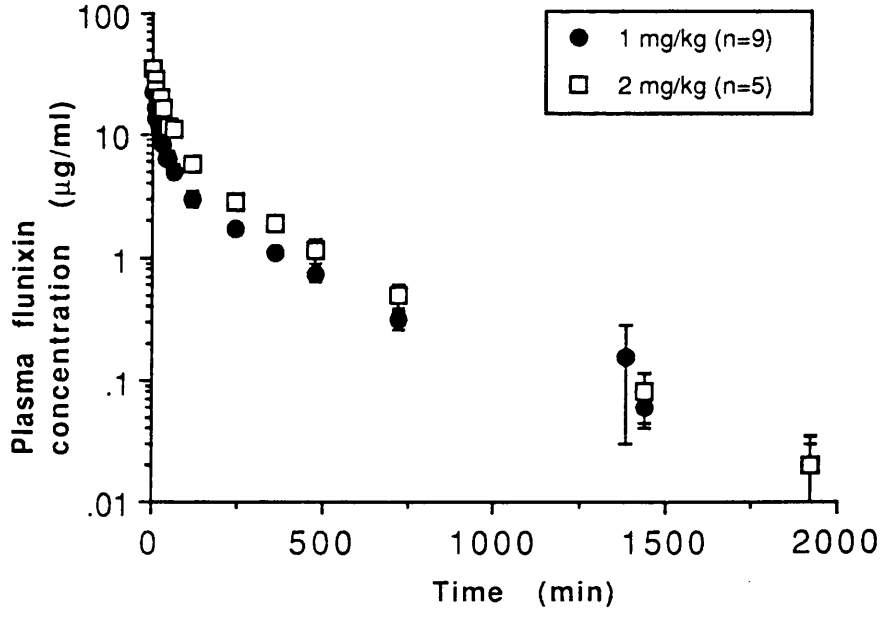

Figure 5.2. A semilogarithmic plot of the decline of mean concentration of flunixin meglumine in plasma (±SEM) of sheep plotted against time in minutes (min) after the IV administration of either 1.0 mg/kg or 2.0 mg/kg of drug.

Sheep Number	P (μg/ml)	A (μg/ml)	B (μg/ml)	π (min^{-1})	α (min^{-1})	β (min^{-1})
1	23.2	13.9	5.77	0.54	0.05	0.006
2	14.7	0.92	0.76	0.04	0.003	0.001
3	17.2	10.1	3.09	0.25	0.01	0.002
4	55.4	13.8	4.78	1.11	0.05	0.003
5	18.3	5.18	2.93	0.05	0.01	0.003
6	16.5	7.21	3.82	0.19	0.009	0.003
7	25.6	7.91	5.11	0.31	0.03	0.004
8	13.6	3.35	2.57	0.08	0.01	0.003
9	11.8	6.84	3.58	0.12	0.01	0.002
Mean	21.8	7.69	3.6	0.30	0.02	0.003
±SEM	4.45	1.46	0.50	0.11	0.006	0.0005

Table 5.2. Individual and mean pharmacokinetic constants, ± standard error of the mean (SEM), for 9 sheep determined after injection of flunixin meglumine, 1.0 mg/kg, IV.

Sheep Number	$t_{1/2\pi}$ (min)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	$Vd_{(area)}$ (ml/kg)	Vc (ml/kg)	$Vd_{(ss)}$ (ml/kg)	kel (min^{-1})	Cl_b (ml/kg.min)
1	1.3	13.6	126.3	123.6	23.3	106.5	0.03	0.7
2	16.1	222.5	728.7	695.4	61.1	457.4	0.01	0.7
3	2.8	60.6	284.6	225.3	32.9	121.5	0.01	0.5
4	0.6	15.3	213.9	182.2	13.5	138.1	0.04	0.5
5	15.1	50.0	222.5	194.6	37.8	114.9	0.02	0.6
6	3.6	76.0	207.2	165.0	36.3	105.3	0.02	0.5
7	2.2	20.9	158.1	168.1	25.9	123.7	0.03	0.7
8	9.0	67.1	226.6	248.8	51.2	171.5	0.02	0.7
9	5.7	48.2	301.6	234.7	44.9	156.8	0.01	0.5
Mean	2.3*	101.0*	221.7*	248.6	36.3	166.2	0.02	0.6
±SEM				57.3	4.9	37.2	0.004	0.03

Table 5.3. The individual and mean plasma pharmacokinetic parameters, ± standard error of the mean (SEM), for 9 sheep given flunixin meglumine, 1.0 mg/kg, IV.

* Harmonic mean.

Whole body clearance, (Cl_b), was slow in all sheep, mean 0.6 ± 0.03 ml/kg.min. The mean residence time of flunixin, 1.0 mg/kg, in plasma for observed values, $MRT_{(obs)}$, was calculated as 189.8 ± 22.3 minutes (Table 5.4).

The mean plasma concentration of flunixin following injection of 2.0 mg/kg of drug, IV, to 5 sheep is shown also in Fig. 5.2. The mean plasma levels showed an initial rapid distribution phase (π phase), followed by a slower second distribution phase (α phase) and a slow elimination phase (β phase). Plots of the plasma flunixin decay curve for each individual sheep were best fitted to a tri-exponential decline and this was confirmed by applying the AIC. Individual values for the rate constants are shown in Table. 5.5. The pharmacokinetic parameters calculated from the tri-exponential equations used to describe the plasma concentration versus time data for individual sheep are shown in Table. 5.6. These indicate an initial rapid distribution half life ($t_{1/2\pi}$) of 1.9 minutes followed by a slower second distribution phase ($t_{1/2\alpha}$) of 40.2 minutes. The mean elimination half-life ($t_{1/2\beta}$) was 205.8 minutes. Sheep number 5 had slow initial and second distribution phases and a slow elimination half-life, 13.6, 81.1 and 302.8 minutes, respectively.

The mean $Vd_{(area)}$ was small, 310.1 ± 54.4 ml/kg, and similarly, the mean values for $Vd_{(ss)}$ and Vc were small, 151.8 ± 26.3ml/kg and 40.9 ± 11.1 ml/kg respectively.

Whole body clearance was slow in all sheep, mean Cl_b was calculated as 0.7 ± 0.01 ml/kg.min. The $MRT_{(obs)}$ of flunixin, 2.0 mg/kg, in plasma was calculated as 201.7 ± 21.7 minutes (Table 5.3).

The mean area under the plasma concentration time curve for observed values (AUC_{obs}) for animals given flunixin, 2.0 mg/kg, IV was approximately 1.95 times greater than the AUC_{obs} for those animals administered 1.0 mg/kg IV, indicating that, between these two dose rates, plasma levels are proportional to the dose rate.

Statistical analysis revealed no significant difference ($p>0.05$) between the kinetic parameters observed at the two different dose rates used in this study.

5.3.2 Assay of carprofen in plasma

Figure 5.3 illustrates a typical chromatograph of a plasma sample collected after administration of carprofen, 0.7 mg/kg, IV. No coextracted endogenous compounds from ovine plasma interfered with the carprofen peak nor was there evidence that any metabolites of carprofen were detected in this system.

Sheep Number	$MRT_{(obs)}$ (min) 1.0 mg/kg	$MRT_{(obs)}$ (min) 2.0 mg/kg
1	141.0	157.2
2	117.0	146.4
3	208.2	226.2
4	207.0	217.8
5	182.4	261.0
6	108.0	
7	209.4	
8	204.0	
9	331.8	
Mean	189.8	201.7
±SEM	22.3	21.7

Table 5.4. The individual and mean, mean residence time for observed values ($MRT_{(obs)}$), ± standard error of the mean (SEM), for sheep given flunixin meglumine, 1.0 or 2.0 mg/kg, IV.

Sheep Number	P (μg/ml)	A (μg/ml)	B (μg/ml)	π (min^{-1})	α (min^{-1})	β (min^{-1})
1	123.6	26.3	3.89	1.09	0.02	0.003
2	12.6	21.8	11.7	0.26	0.03	0.006
3	11.2	18.0	3.31	0.10	0.01	0.002
4	31.27	14.2	8.09	0.30	0.02	0.004
5	16.0	4.84	4.05	0.05	0.009	0.002
Mean	38.9	17.0	6.21	0.36	0.02	0.003
±SEM	21.4	3.70	1.61	0.19	0.003	0.0008

Table 5.5. Individual and mean pharmacokinetic constants, ± standard error of the mean (SEM), for 5 sheep determined after injection of flunixin meglumine, 2.0 mg/kg, IV.

Sheep Number	$t_{1/2\pi}$ (min)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	$Vd_{(area)}$ (ml/kg)	Vc (ml/kg)	$Vd_{(ss)}$ (ml/kg)	kel (min^{-1})	Cl_b (ml/kg.min)
1	0.6	42.9	201.5	232.2	13.0	104.3	0.05	0.7
2	2.6	21.6	125.8	467.8	43.3	100.1	0.02	0.7
3	6.8	55.8	334.3	317.0	30.8	178.4	0.01	0.6
4	2.3	39.5	196.7	155.9	37.3	135.6	0.02	0.6
5	13.6	81.1	302.8	377.4	80.3	240.7	0.01	0.8
Mean	1.9*	40.2*	205.8*	310.1	40.9	151.8	0.02	0.7
±SEM				54.4	11.1	26.3	0.007	0.01

Table 5.6. The individual and mean plasma pharmacokinetic parameters, ± standard error of the mean (SEM), for 5 sheep given flunixin meglumine, 2.0 mg/kg, IV.

* Harmonic mean.

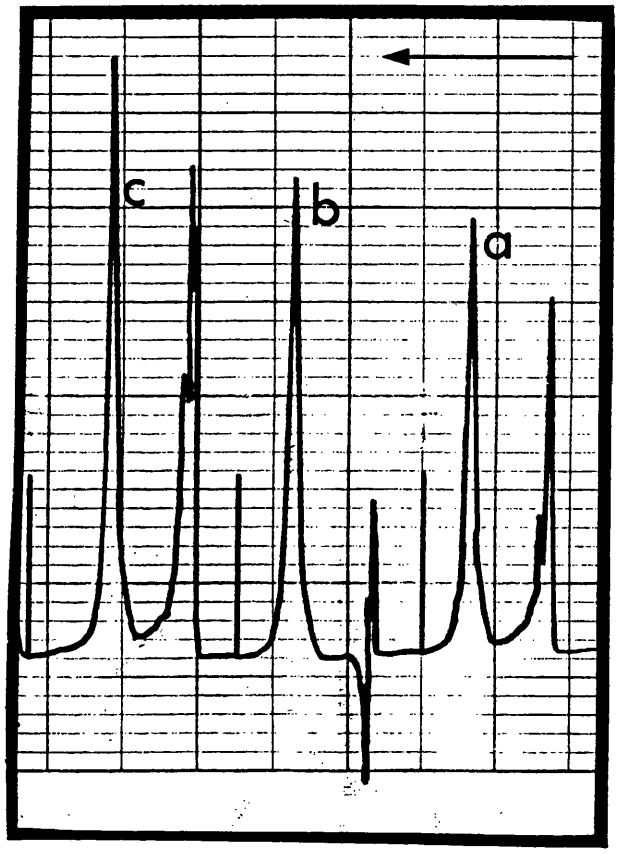

Figure 5.3. Typical chromatogram of carprofen in ovine plasma.

Key- **a-** 5 µg/ml carprofen in ovine plasma (standard injection)

b and **c-** unknown ovine plasma samples

→- direction of flow

The precision of the extraction procedure was good as can be seen from the between-day coefficients of variation (Table 5.7). The mean between-day coefficient of variation was 11.7 %. No carprofen was detected in the drug free plasma.

The mean plasma concentration of carprofen, 0.7 mg/kg, following IV injection to 5 sheep plotted against time (min), is shown in Fig. 5.4. The mean plasma levels showed an initial rapid decline (α phase) and a slower elimination phase (β phase). Plots of the plasma concentration decay curve for each individual sheep were best fitted to a bi-exponential decline and this was confirmed by applying the AIC. The individual values for the rate constants are shown in Table. 5.8. The pharmacokinetic parameters calculated from the bi-exponential equations used to describe the plasma concentration versus time data for individual sheep are shown in Table. 5.9. The mean elimination half-life ($t_{1/2\beta}$) was 25.8 hours with a range from 21.9 to 28.5 hours.

The mean plasma disposition and pharmacokinetic characteristics indicate a small volume of distribution, $Vd_{(area)}$, 95.5 ± 5.95 ml/kg, a small volume of distribution at steady state, $Vd_{(ss)}$, 92.7 ml/kg and a slow whole body clearance, Cl_b, 2.5 ± 0.1 ml/kg.hr. The specific volume of the central compartment, Vc, ranged from 44.6 to 64.7 ml/kg. The mean residence time of carprofen, 0.7 mg/kg, in plasma for observed values, $MRT_{(obs)}$, was calculated as 17.4 ± 2.06 hours (Table 5.10).

The mean plasma concentration of carprofen, 4.0 mg/kg, following IV injection in 5 sheep plotted against time (min), is shown in Fig. 5.4. The mean plasma levels showed an initial rapid decline (α phase) and a slower elimination phase (β phase). Plots of the plasma concentration decay curve for each individual sheep were best fitted to a bi-exponential decline and this was confirmed by applying the AIC. The individual values for the rate constants are shown in Table. 5.11. The pharmacokinetic parameters calculated from the bi-exponential equations used to describe the plasma concentration versus time data for individual sheep are shown in Table. 5.12. The mean elimination half-life ($t_{1/2\beta}$) was 32.3 hours with a range from 24.6 to 43.5 hours.

The mean plasma disposition and pharmacokinetic parameters indicate a small $Vd_{(area)}$, 117.3 ± 3.53 ml/kg, small $Vd_{(ss)}$, 117.3 ml/kg and a slow Cl_b, 2.5 ± 0.3 ml/kg.hr. The Vc ranged from 52.5 to 67.9 ml/kg. The $MTR_{(obs)}$, (Table 5.8) of carprofen, 4.0 mg/kg, in plasma was calculated as 27.2 ± 1.66 hours. Statistical analysis indicated that the $MTR_{(obs)}$ for carprofen, 4.0 mg/kg, IV, was significantly longer than for carprofen, 0.7 mg/kg, IV ($p \leq 0.05$) (Table 5.10).

Concentration (μg/ml)	Mean recovery (%)	Standard deviation	Coefficient of Variation (%)
0.25	80.9	16.2	20.0
0.5	98.1	12.3	12.5
1.0	92.6	6.1	6.6
2.0	105.8	13.2	12.5
5.0	97.7	6.7	6.9
Mean	95.0	10.9	11.7

Table 5.7: Mean recovery and coefficient of variation (%) of carprofen in ovine plasma (n=10).

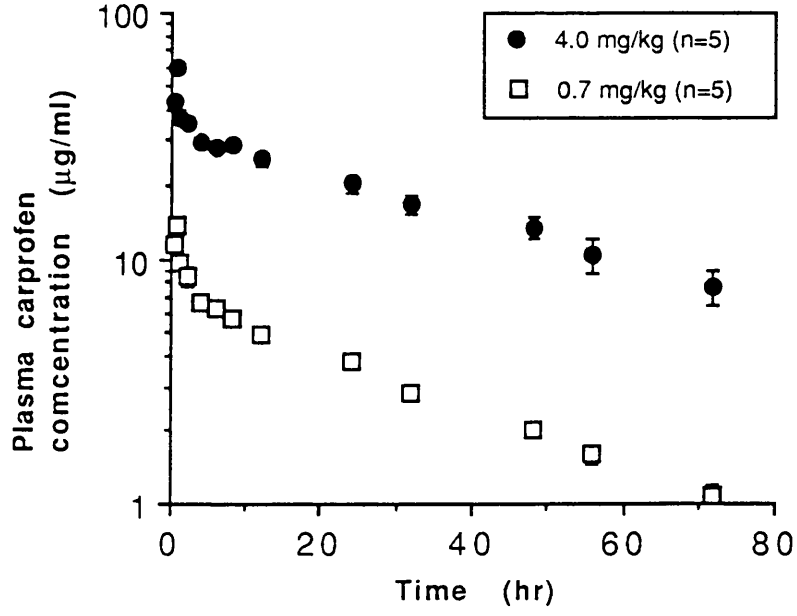

Figure 5.4. A semilogarithmic plot of the decline of mean plasma concentration (±SEM) of carprofen racemate, in sheep, with time in hours (hr), after the IV administration of either 0.7 mg/kg or 4.0 mg/kg of drug.

Sheep Number	A (μg/ml)	B (μg/ml)	α (hr^{-1})	β (hr^{-1})
1	5.91	4.91	0.57	0.03
2	8.55	5.35	0.50	0.03
3	7.77	7.58	1.28	0.02
4	8.38	7.33	0.74	0.03
5	7.77	6.66	1.21	0.03
Mean	7.8	6.37	0.86	0.03
±SEM	0.47	0.53	0.16	0.002

Table 5.8. Individual and mean, ± standard error of the mean (SEM), pharmacokinetic constants for 5 sheep determined after IV injection of carprofen, 0.7 mg/kg.

Sheep Number	$t_{1/2\beta}$ (hr)	$Vd_{(area)}$ (ml/kg)	Vc (ml/kg)	$Vd_{(ss)}$ (ml/kg)	kel (hr^{-1})	Cl_b (ml/kg.hr)
1	26.3	114.0	64.7	110.0	0.05	3.0
2	21.9	78.8	50.4	76.0	0.05	2.5
3	28.5	90.6	45.6	88.9	0.05	2.2
4	26.0	91.7	44.6	88.2	0.06	2.4
5	27.7	102.6	48.5	100.2	0.05	2.6
Mean	25.8*	95.5	50.8	92.7	0.05	2.5
±SEM		5.95	3.63	5.77	0.002	0.1

Table 5.9. The individual and mean, ± standard error of the mean (SEM), plasma pharmacokinetic parameters for 5 sheep given carprofen, 0.7 mg/kg, IV.

* Harmonic mean.

Sheep Number	MRT(obs) (hr) 0.7 mg/kg	MRT(obs) (hr) 4.0 mg/kg
1	16.3	25.0
2	12.0	29.0
3	14.4	23.0
4	22.8	26.4
5	21.5	32.6
Mean	17.4	27.2*
±SEM	2.06	1.66

Table 5.10. The individual, mean, ± standard error of the mean (SEM) mean residence times for observed values (MRT(obs)) for sheep given carprofen, 0.7 or 4.0 mg/kg, IV.

*$p \leq 0.05$ significantly different from 0.7 mg/kg treatment group.

Sheep Number	A (μg/ml)	B (μg/ml)	α (hr^{-1})	β (hr^{-1})
1	34.9	34.6	7.56	0.02
2	31.4	27.5	2.19	0.03
3	34.8	30.9	1.50	0.02
4	34.8	30.5	1.63	0.02
5	39.8	36.3	2.92	0.02
Mean	35.1	32.0	3.16	0.02
±SEM	1.34	1.56	1.13	0.002

Table 5.11. Individual and mean, ± standard error of the mean (SEM), pharmacokinetic constants for 5 sheep determined after injection of carprofen, 4.0 mg/kg, IV.

Sheep Number	$t_{1/2\beta}$ (hr)	$Vd_{(area)}$ (ml/kg)	Vc (ml/kg)	$Vd_{(ss)}$ (ml/kg)	kel (hr^{-1})	Cl_b (ml/kg.hr)
1	30.3	115.4	57.6	115.0	0.05	2.6
2	24.6	125.9	67.9	124.5	0.05	3.5
3	33.8	127.4	62.7	125.6	0.04	2.6
4	36.4	113.7	61.2	112.6	0.04	2.2
5	43.5	109.4	52.5	108.8	0.03	1.7
Mean	32.3*	118.4	60.4	117.3	0.04	2.5
±SEM		3.53	2.57	3.31	0.004	0.3

Table 5.12. The individual and mean, ± standard error of the mean (SEM), plasma pharmacokinetic parameters for 5 sheep given carprofen, 4.0 mg/kg, IV.

* Harmonic mean.

The mean area under the plasma concentration time curve for observed values ($AUC_{(obs)}$) for sheep given carprofen, 4.0 mg/kg, IV, was approximately 5.7 times greater than the $AUC_{(obs)}$ for those animals administered 0.7 mg/kg, IV, indicating that, between these two dose rates, plasma levels are proportional to the dose rate.

With the exception of the $MRT_{(obs)}$, statistical analysis revealed no significant difference ($p>0.05$) between the kinetic parameters observed at the two different dose rates used IV in this study. It is interesting to note that an increase in plasma carprofen concentration occurred between 6 and 8 hours after drug administration at both dose rates.

5.4 Discussion

5.4.1 Assay of flunixin meglumine in plasma

The decline in the plasma concentration of flunixin meglumine with time, after a single IV injection of either 1.0 or 2.0 mg/kg bodyweight, was best described by a triexponential equation in all sheep. This differs from the kinetic studies in cattle, 1.1 mg/kg (Benitz, 1984; Hardee, *et al.* , 1985; Anderson *et al.* , 1990) horses, 1.0 mg/kg (Chay *et al.* , 1982) and dogs, 1.1 mg/kg (Hardie *et al.*, 1985b), where the plasma concentration decay curve of flunixin was best fitted by a two compartment model, although Chay *et al.* (1982), suggested a possible third compartment for flunixin in horses. Soma *et al.* (1988) confirmed the presence of a third compartment in the horse after administration of a higher dose rate of 2.2 mg/kg. Compartmental modelling was not carried out on the data from the present study, but it is likely that the third exponent detected in this study represents transfer of drug out of a third compartment. The detection of a third exponent in this study, at both dose rates, is probably related to the frequency and duration of sampling and to assay sensitivity. Residue studies on flunixin meglumine in sheep have not been carried out to date, and consequently, this may have implications for the use of flunixin in the sheep, a food producing animal, although the terminal elimination half-life of the drug in sheep was not prolonged. However, it is interesting to note that the administration of flunixin, 1.1 mg/kg, intramuscularly every 8 hours, over a period of 60 hours, to lactating cattle did not lead to accumulation of the drug in plasma (Anderson *et al.* , 1990).

There were no significant differences between the kinetic parameters for flunixin, 1.0 and 2.0 mg/kg, administered IV. The mean initial distribution half-lives of flunixin in sheep, 2.3 and 1.9 minutes, are less than those reported by Anderson *et al.* (1990) in lactating adult cattle (9.6 min), and also less than those reported in the horse, 12 minutes (Chay *et al.* , 1982) and dog, 33 minutes (Hardee *et al.* ,1985b). In these previous studies, plasma concentration decay curves of flunixin were best fitted by a two compartment model and it is possible that the distribution half-lives quoted lie between the values found in the present study for the initial and second distribution half-lives *i. e.* 2.3 and 1.9, and 101.0 and 40.2 minutes respectively. The mean elimination half-lives ($t_{1/2\beta}$) of flunixin in this study, 229.8 and 205.8 minutes, are similar to those reported for lactating cattle, 188.4 minutes (Anderson *et al.*, 1990) and also dogs, 220.2 minutes (Hardie *et al.*, 1985b). However, Hardee *et al.* (1985), reported a $t_{1/2\beta}$ in cattle of considerably longer duration than those already mentioned, 487.2 minutes. The assay for flunixin used by the latter authors was less sensitive than the assay used in this study, resulting in a five fold decrease in the limit of detection achieved (50 ng/ml *vs* 10 ng/ml). Although Hardee *et al.*

(1985) sampled for 72 hours after the administration of flunixin, plasma concentrations of the drug fell below the limit of detection by 12 hours. The differences between the sensitivities of the assays, limiting the time over which the drug could be detected may explain the differences seen in $t_{1/2\beta}$. In comparison the $t_{1/2\beta}$ reported for horses (Chay *et al.*, 1982) is of much shorter duration, in the order of 96 minutes, which is comparable with that proposed for the cat (Taylor *et al.*, 1991). However, in the latter study insufficient data points precluded the accurate calculation of this parameter. The half-life values obtained in the present study have been expressed as the harmonic mean, while the authors cited above refer to the arithmetic mean. The harmonic mean is the most appropriate average to use when dealing with rates (Moroney, 1963). It is worth noting that sheep number 2, after the administration of 1.0 mg/kg of flunixin, had a particularly long $t_{1/2\beta}$, 728.7 minutes. This was a consequence of a large volume of distribution, $V_{d(area)}$, 695.4 ml/kg.

The small value for the volume of distribution of flunixin, at both dose rates, illustrated that the drug was not widely distributed throughout the body. This is perhaps unsurprising since NSAIDs are all highly protein bound in plasma (Lees *et al.* , 1991a).

The mean body clearance rates of flunixin in sheep were 0.6 and 0.7 ml/kg.min, less than those previously reported in cattle (Hardee *et al.*, 1985; Anderson *et al.*, 1990), horses (Soma *et al.*, 1988) and dogs (Hardie *et al.*, 1985b). This suggests that sheep may clear flunixin at a slower rate than other species.

The estimation of cyclooxygenase inhibition by flunixin in sheep would have provided further information on the duration of action of the drug in this species. The generation of the stable breakdown product of platelet thromboxane (TX) A_2 in serum, TXB_2, has been used to investigate the level of cyclooxygenase inhibition after the administration of NSAIDs (Vane, 1971; Lees *et al.* , 1987a and b). Serum levels of flunixin >2 μg/ml in dogs have been shown to inhibit serum thromboxane synthesis by more than 98% *in vivo*, and in dogs and horses by more than 99% *in vitro* (McKellar *et al.* , 1989). Soma *et al.* (1992) demonstrated that plasma levels of flunixin of approximately 0.12 μg/ml were not associated with the inhibition of serum thromboxane synthesis in the horse, while Semrad *et al.* (1985) demonstrated that plasma levels of >75 μg/ml were associated with 100 % inhibition of serum TXB_2, in this species. Similarly, Lees and Taylor (1991) found the inhibition of serum TXB_2 could only be demonstrated for approximately 5 hours after the administration of 1.0 mg/kg, IV, in the cat. The mean plasma level of flunixin at 5 hours in this latter study was approximately 0.2 μg/ml. After IV administration of flunixin, 1.0 or 2.0 mg/kg, the drug was only detected in ovine plasma in the present study at levels >2 μg/ml, for approximately 120 minutes, while the mean

plasma concentration persisted between 0.12 and 0.2 μg/ml for up to 720 minutes. McKellar *et al.* (1990a) reported that sheep have low baseline values of TXB_2 and consequently, neither the extent or the duration of cyclooxygenase inhibition were measured in this study.

Lees and Higgins (1984) showed, in a tissue model of equine inflammation, that flunixin meglumine will suppress prostaglandin E_2-like activity for at least 24 hours, longer than flunixin can be detected in the plasma. These findings were supported by further studies from the same laboratory in both the horse and the cow (Lees *et al.* , 1987a; Lees, 1989). These findings highlighted that information on the penetration into, and the persistence of the NSAIDs in inflammatory exudate is perhaps more valuable with regard to establishing dosage regimes, than is pharmacokinetic data (Lees,1992).

5.4.2 Assay of carprofen in plasma

The decline in plasma concentration of carprofen racemate with time, after a single IV injection of either 0.7 mg/kg or 4.0 mg/kg bodyweight, was best described by a bi-exponential equation in all sheep. However, the decline in plasma concentration of carprofen, 100 mg, IV, with time, in humans (Crevoisier, 1982), and after the IV injection of 1.0 mg/kg in dogs (Rubio *et al.* , 1980), was best described by a three compartment kinetic model. It is possible that a second phase of distribution also occurred in sheep but infrequency of sampling in the immediate post-injection period precluded the detection of this phase. The high drug concentration of carprofen in ovine plasma, at both 0.7 and 4.0 mg/kg, may be explained by the small volume of distribution, 95.5 ±5.95 and 118.4 ±3.5 ml/kg, respectively, illustrating that the drug was not widely distributed throughout the body. These results are consistent with those reported in the horse (McKellar *et al.*, 1991c), dog (McKellar *et al.*, 1990b) and cow (Ludwig *et al.*, 1989), and are probably related to the high degree of protein binding of carprofen, which can be as great as 99.9 % at therapeutic concentrations (Crevoisier, 1982; Baruth *et al.*, 1985), as with most other NSAIDs.

There were no significant differences between the kinetic parameters for 0.7 and 4.0 mg/kg carprofen racemate administered IV. The distribution half-life of carprofen in plasma was not calculated in the present study because of the small number of samples that were taken in the first hour after drug administration. Calculation of this parameter from the available information therefore would be inaccurate, and indeed the quoted values for calculated pharmacokinetic parameters which use the α rate constant or A, the zero-time plasma drug concentration intercept, may be inaccurate. The elimination half-life of carprofen in sheep was 25.8 and 32.3 hours after the administration of 0.7 and 4.0 mg/kg, IV, respectively. These values were similar to those observed in the horse and

pony administered 3.5 mg/kg, IV, (21.9 ± 2.3 hr) (McKellar *et al.*, 1991c). In the lactating cow the plasma elimination half-life ranged from 44.5 to 64.6 hours (Ludwig *et al.*, 1989), considerably longer than that observed in sheep in the present study, and over three times greater than that observed by McKellar *et al.* (1990b) in dogs administered carprofen, 0.7 mg/kg, IV, (8.0 ± 1.2 hr). It is possible that the existence, or differences in the degree of enterohepatic circulation of carprofen between species could account for the wide range of values seen.

It appears likely that enterohepatic recycling occurs between 6 and 8 hours in sheep (Fig. 5.4). An increase in plasma carprofen concentration occurred in four of the five sheep sampled. Enterohepatic circulation of carprofen has been described both in humans (Ray and Wade, 1982) and the dog (McKellar *et al.* , 1990b), although Rubio *et al.* (1980) did not detect any evidence of enterohepatic circulation of carprofen in the dog in an earlier study.

Whole body clearance of carprofen, 2.5 ml/kg.hr, was slower than reported in cattle (Ludwig *et al* , 1989), dogs (McKellar *et al.*, 1990b) and horses (McKellar *et al.*, 1991c). This may be because sheep clear NSAIDs at a slower rate than do other species, as was reported in an earlier study by this laboratory (Welsh *et al.*, 1993). However, enterohepatic recycling also may influence the rate at which carprofen is cleared in sheep and this is supported by the prolonged mean residence time of observed values of carprofen in the body.

Carprofen is presented in solution as a 50:50 racemic mixture, and in general, enantiomers have similar physical and chemical properties although their physiological effects may differ. When racemic compounds are used therapeutically, the more active isomer is referred to as the eutomer and the less active isomer as the distomer (Ariens, 1986). Therapeutic agents which are marketed as the racemate, including carprofen, ketamine and ketoprofen, may contain up to 50 % of the inactive enantiomer. Ariens (1986) pointed out that the presence of the distomer in therapeutic agents could be disadvantageous for several reasons *e. g.* the distomer may act as a competitive antagonist, or partial agonist, at the drug receptor; the isomers may be physiological antagonists, resulting in opposite biological effects and finally, the distomer may cause side effects not shown by the eutomer. However, it should be noted that distomers may be equipotent with the eutomer, or the distomer may contribute to the efficacy of the eutomer *e. g.* the distomer may reduce the metabolic degradation of the eutomer such as occurs with the analgesic levomethorphan, with co-administration of the distomer dextromethorphan (Cooper and Anders, 1974). The physiological effects of the carprofen enantiomers differ markedly, and the S(+)-isomer is a more potent anti-inflammatory

agent than the R(-)-isomer. However, the distomer of carprofen is not inactive and has been shown to have greater anti-inflammatory activity than phenylbutazone and aspirin (Gaut *et al.* , 1975; Randall and Baruth, 1976). Currently, there is no evidence to suggest that the activity of the S(+)-isomer of carprofen relies on the presence of the distomer, nor that the distomer of carprofen acts as an antagonist to the more active S(+)-isomer.

It is apparent that enantiomers can exert different effects on biological systems and therefore, it is not unreasonable to assume that enantiomers may be handled differently by biological systems during absorption, distribution, metabolism and excretion. Stereoselectivity appears to be of greatest importance in relation to protein binding and hepatic metabolism of isomers, with drug absorption and renal excretion being affected to a lesser degree (Williams and Lee, 1985). Obviously, such differences may alter calculated pharmacokinetic parameters for the 2 isomers. Moreover, it is important to establish whether chiral inversion of the eutomer or the distomer occurs within the body. Although, chiral inversion of the 2-arylpropionic acids, including carprofen, has been demonstrated (Hutt and Caldwell, 1984), chiral inversion of carprofen enantiomers did not occur in dogs despite differing pharmacokinetic profiles (McKellar, personal communication), and similarly, negligible inversion R(-) to S(+), or *vice versa*, occurred in humans (Stoltenberg *et al.* , 1981; Iwakawa *et al.* , 1989). Consequently, when a therapeutic agent is administered to an animal as the racemate, the pharmacokinetics of the 2 isomers should be examined. Chromatographic separation of enantiomers may be achieved either by using a chiral chromatographic system (Graser *et al.* , 1991) or by derivitisation of the compound prior to separation by standard chromatographic techniques (Sallustio *et al.* , 1986). In the present study the pharmacokinetics of racemic carprofen only are presented, however, further analysis of is being undertaken currently to elucidate the disposition of the individual enantiomers of carprofen in sheep plasma using the method described by Sallustio *et al.* (1986).

In conclusion, the results of the present study indicate that accumulation of flunixin meglumine, 1.0 or 2.0 mg/kg, IV, should not occur if the drug is administered at 12 or 24 hour intervals, and that a dose rate of 1.0 mg/kg would be appropriate for further investigation in sheep. Similarly, a dose rate of flunixin meglumine, 1.1 mg/kg, intramuscularly, has been recommended in sheep (Welsh *et al.* , 1993), and it was shown that accumulation of flunixin meglumine, 2.2 mg/kg, intramuscularly, did not occur after repeat injection at 24 hour intervals in the same study. The authors in the latter study demonstrated that absorption of flunixin from the intramuscular injection site occurred rapidly, and that bioavailability of the drug was high. In addition, the results of the present study also indicate that carprofen, 0.7 mg/kg, IV, would be an appropriate dose rate for further studies in sheep. Further investigations of the bioavailability and

pharmacokinetics after intramuscular injection of carprofen in sheep would be valuable because this route of administration is used frequently in the larger domestic species.

CHAPTER 6

METHODS OF LAMENESS MEASUREMENT

6.1 Introduction

Lameness in animals occurs when there is a disturbance of the normal gait pattern during locomotion. The animal may be lame either as a result of pain in one of the body systems involved in locomotion, or as a result of mechanical interference with normal motion (Stashak, 1987; Scott, 1989). Frequently, however, both of these factors play a role in lameness *e. g.* interdigital fibroma in rams (Bulgin, 1986).

Normal gait and lameness can be assessed both subjectively and objectively. One of the simplest ways of evaluating lameness clinically is subjective assessment based on observation of factors such as alterations in stride length, duration of weight bearing on affected and unaffected limbs, carriage of the limb, head and tail position and joint movement (Leach *et al.* ,1977). Such details may be recorded to allow comparison not only between animals, but also of the same animal on subsequent occasions. However, there are also various methods of objective assessment of both normal and abnormal gait although these are used more commonly experimentally because of the high costs of many of these techniques (Nunamaker and Blauner, 1985).

6.1.1 Subjective rating scales

Rating scales are used frequently in scoring subjective phenomena. Pain and signs of pain are some of the most difficult subjective clinical parameters for both medical (Banos *et al.* , 1989) and veterinary clinicians (Taylor, 1982) to quantify. Pain is a subjective multidimensional experience with both sensory and affective considerations (Prieto and Geisinger, 1983) and medical clinicians must rely upon observation and patient report to evaluate it (Revill *et al.*, 1976). Similarly, in veterinary medicine the clinician must rely upon their own evaluation of the animal and on owners reports of signs in the animal in order to assess pain. Consequently, many subjective rating scales have been designed for use in the assessment of pain and have undergone extensive evaluation. Indeed, subjective rating scales are used widely where objective assessments of the problem under consideration are difficult or impossible to obtain. However they have been used in situations where quantitative analysis of the condition may also be available *e. g.* airway obstruction (Swinburn *et al.* , 1984) and post-operative swelling (Berge, 1988).

6.1.1.1 Simple descriptive scale

The simple descriptive scale (SDS) or verbal rating scale (VRS) as it is also known, provides a choice of verbal descriptions, often between 4 and 5, of the problem under consideration *e. g.* pain (Downie *et al.*, 1978a), and thus it forces the translation of feeling into words (Ohnhaus and Adler, 1975). Keele (1948), for example selected

words commonly used by patients to describe their own pain to construct the following scale; nil, slight, moderate, severe and agonising. The SDS treats change as a unit step (Keele, 1948; Lasagna, 1964) and as such is simple to use for scoring pain subjectively (Keele 1948; Huskisson, 1974). However, it has been demonstrated that there are unequal distances between verbal descriptions of pain, which frequently are considered to be equidistant (Sriwatanakul *et al.* , 1982), and as many as 39 different levels of pain have been perceived between the point when pain is first detected *i. e.* the pain threshold, and that point at which pain can no longer be tolerated *i. e.* the pain tolerance (Hardy *et al.* , 1952, Grossi *et al.* , 1983). Consequently, the SDS lacks sensitivity, not only because an assumption is made that the differences between each term used are equal (Huskisson, 1974; Ohnhaus and Adler, 1975; Scott and Huskisson, 1976; Downie *et al.* , 1978a), but also because a very limited number of words are used to describe pain in the scale. In practice, this assumption does not allow for small changes in scoring and Ohnhaus and Adler (1975) showed that the use of a SDS artificially increased the analgesic effect of a drug. The SDS is usually transformed by applying numbers to each term, thus nil = 0, slight = 1, *etc.* in order to quantify the scale and to convert it from a nominal to an ordinal scale (Deschamps *et al.* , 1988). However, in doing this, the assumption that the differences between each term are equal is compounded, and as such the variations seen in scores cannot be expressed mathematically (Aitken, 1969).

6.1.1.2 Numerical rating scale

Numerical rating scales (NRS) are perhaps the most frequently used rating scales for subjective assessment in veterinary medicine. The NRS consists of divisions which are represented by numerical values *e. g.* 0 to 10 or 0 to 100. Those using the scale assign a numerical value to the condition under investigation *e. g* lameness or pain, that they feel represents the level of severity of that condition (Fordyce *et al.* , 1984). Numerical rating scales have been used to record body condition scores in a variety of species (Henneke *et al.* , 1983; Braun *et al.* , 1986), as well as carcase fat thickness (Lee *et al.* , 1984). These scales also have been used to rate lameness in many large animal species including, sheep (Ley *et al.* , 1989), cattle (Braun *et al.* , 1987) and horses (Stashak, 1987; Wyn-Jones, 1988). In addition, signs of pain in dogs have been scored using an NRS by many workers (Taylor and Houlton, 1984; Taylor and Herrtage, 1986; Waterman and Kalthum, 1988; Thompson and Johnson, 1991). Numerical rating scales provide an improvement of discrimination when compared to the SDS (Downie *et al.* , 1978a; Downie *et al.* , 1978b), but still lack sensitivity when compared to other methods such as the visual analogue scale (Huskisson, 1983). However, Downie *et al.* (1978a) found that the NRS provided the best balance between the SDS, which limits choice, and the visual analogue scale (*vide infra*) which is confusing due to the lack of limitations. Again, the

assumption is made that the numerical divisions represent an equal increase, or decrease in the condition under investigation.

6.1.1.3 Visual analogue scale

The visual analogue scale (VAS) (Hayes and Paterson, 1921; Freyd, 1923), uses a straight line, usually 100 mm long, the extreme limits of which are marked with perpendicular lines. The VAS may be vertical or horizontal (Huskisson, 1974; Revill *et al.* , 1976; Scott and Huskisson, 1979; Langley and Sheppeard, 1984). Both ends of the scale have a verbal description of each extreme of the clinical sign or symptom to be evaluated, and the observer is asked to mark the line at a position that represents the severity of the sign or symptom under investigation. The verbal descriptions at the extremes of the scale are intended to anchor the ends of the scale so that the intermediate points may be assigned more easily (Joyce *et al.* ,1975). Data are generally converted to numerical form by measurement of the distance to where the mark is made. Visual analogue scales have proved particularly satisfactory for subjective measurement of pain (Scott and Huskisson, 1976), or relief of pain in human beings (Huskisson, 1974) and they are used extensively for this purpose (Carlsson, 1983). The VAS also has been applied for measurement of feelings (Aitken, 1969), thirst (Thompson *et al.* , 1987; Thompson *et al.* , 1991) and certain psychiatric disorders such as anxiety (McClelland *et al.* , 1977; Ansseau and Von Frenckell, 1991) and depression (McClelland *et al.* , 1979; Faravelli *et al.* , 1986). Clinical parameters that can be assessed easily using objective techniques, such as breathlessness (Swinburn *et al.*, 1984) and post-operative facial swelling (Berge, 1988), also have been evaluated using the VAS. The VAS has been found to increase the sensitivity of measurement of pain over other rating scales (Huskisson, 1974; Joyce *et al.* , 1975; Ohnhaus and Adler, 1975; Scott and Huskisson, 1976; Seymour, 1982).

There has been much discussion as to whether trained observers should be used to score pain in human patients, rather than allowing patients to record their own pain scores using a VAS (Parkhouse and Holmes, 1963). The luxury of such choice is not available to veterinary surgeons, and all assessments of pain, discomfort, lameness or any subjective measurement in animals must be made by clinicians or trained observers. The use of the VAS in veterinary medicine has been limited to date (Mburu, 1991; Reid and Nolan, 1991). Reid and Nolan (1991) recently reported the use of the VAS for scoring post-operative signs of pain and sedation in dogs, and commented that previously, verbal descriptive scales have been most commonly used for this purpose in veterinary medicine. Pain is a composite measurement and one that is very difficult to quantify. Given that the application of the VAS for this purpose has been suggested to be superior

to other methods of subjective measurement (Joyce *et al.* , 1975; Ohnhaus and Adler, 1975), it might be expected that use of a VAS for measurement of other composite subjective responses, such as lameness could be equally advantageous in increasing the sensitivity of measurement.

6.1.1.4 Graphic rating scale

Graphic rating scales (GRS) are an amalgam formed from the simple descriptive scale and the visual analogue scale (Freyd, 1923). The GRS consists of a VAS with descriptive terms placed at intervals along the length of the scale (Scott and Huskisson, 1976). Assigning descriptive terms along the length of the VAS, thus converting it to a GRS, has been found to make the scale easier to use, especially if those people scoring are unfamiliar with rating scales in general (Scott and Huskisson, 1976; Heft and Parker, 1984; Reid and Nolan, 1991). As with the VAS, there are an infinite number of points which can be assigned along the length of the scale (Huskisson 1974). However, the responses recorded by those using the GRS are frequently clustered near the descriptive terms on the scale (Huskisson 1974; Heft and Parker, 1984), effectively limiting the sensitivity of the GRS to that of the SDS (Huskisson 1974; Scott and Huskisson, 1976). Huskisson and Scott (1977) found that this particular problem of clustering of results rendered the GRS unsatisfactory when used to rate pain relief. Freyd (1923) commented on the possibility of changing the interval between descriptive phrases to compensate for abnormal distribution of scores in a study on personality traits. Similarly, Heft and Parker (1974) suggested that the intervals between the words on the GRS should reflect the intervals between the words as those scoring perceived them, with moderate being rated as 0 and responses below that rated as negative and responses above moderate rated as positive. Moderate would be rated as 0 because the distance between the lowest description and no pain cannot be evaluated. A further problem which they noted, was that some patients used points beyond the extremes of the scale, and in conclusion, the authors proposed that uneven intervals between the descriptive terms employed along the GRS should be used to overcome the problem of clustering.

6.1.2 Objective gait analysis

The study of gait in man and animals is well established although it remains difficult to gain useful diagnostic or therapeutic benefit from the data (Nunamaker and Blauner, 1985). Gait analysis can provide not only information about the geometry of movement (kinematics), and the timing of movement (temporal information), but also about the forces that initiate, maintain and alter movement (kinetics). Recent work has concentrated on the horse because locomotive disorders are a major source of economic loss to the

racing industry (Fredricson *et al.* , 1980). A brief review of objective methods of lameness assessment will be given.

In the late 19th century, Eadweard Muybridge was accredited with pioneering the use of moving pictures, using stroboscopic photography, to record the gait of animals such as the horse and greyhound, and subsequently, many workers have contributed to this field. Leach and Dagg (1983) provide an excellent review of this work. Currently, high speed cinematography provides a method of gait analysis which allows both temporal and kinematic aspects of locomotion to be evaluated (Fredricson *et al.* , 1970; 1972; 1974; 1976; 1980; Fredricson and Drevemo, 1971; 1972a; 1972b; 1972c). This technique documents movement accurately and in a standardized manner to provide a visual record of motion (Leach, 1987). Although an extremely expensive method of analysis, high speed cinematography can provide a vast array of information with each frame, and this can be stored easily for later analysis (Fredricson *et al.* , 1980; Leach, 1987). Although most research in this field has been directed towards the characterisation of normal gaits, some studies have used the technique to characterise gait patterns in lame animals (Clayton, 1986a; 1986b; 1988; Ratzlaff *et al.* , 1989).

Force plates measure ground reaction forces (Fackelman and Seeherman, 1983), and are manufactured with strain gauges, or piezoelectric quartz transducers at each corner (Fackelman and Seeherman, 1983; Leach, 1987). They record the magnitude, direction, duration and position of the foot as it is placed on the plate (Leach, 1987), thus allowing evaluation of temporal and kinetic aspects of movement, and they have been used in man (Cavagna, 1975), horses (Pratt and O'Connor, 1976; Steis *et al* , 1982), cattle (Scott, 1988; 1989), cats (Coulmance *et al.* , 1979) and dogs (Dueland *et al.* , 1977; Budsberg, 1987; Budsberg *et al.* , 1987; 1988). Analysis of force plate data has been used as a reliable method of quantifying limb dysfunction and response to medical and surgical intervention, not only in horses (Gingerich *et al.* , 1979; 1981; Auer *et al.* , 1980; Merkens and Schamhardt 1988a; 1988b; Dow *et al.* , 1991), but also in dogs (Dueland *et al.* , 1977, Budsberg *et al.* , 1987, Person, 1989, McLaughlin *et al.* , 1991) and cattle (Scott 1989).

Goniometry is the objective measurement of joint motion or joint position (Nicol, 1989), and Smith (1982) has reviewed the development of goniometry in human medicine. Using electrogoniometry, joint characteristics such as range and amplitude of motion can be recorded (Leach, 1987), and studies have been made not only of normal joint motion in dogs and horses (Adrian *et al.* , 1966; Taylor *et al.* , 1966), but also in animals suffering lameness (Adrian *et al.* , 1977; Ratzlaff *et al.* , 1982; Ratzlaff and Grant, 1986).

They also have been used for quantitative evaluation of drug treatment on locomotion (Ratzlaff *et al.*, 1981).

Although cinematography, force plate analysis and electrogoniometry are perhaps the most frequently reported techniques of objective lameness assessment, there are other techniques which provide objective data and have proven useful either in their own right, or in association with other techniques. These methods include, pedobarographs (Scott, 1988), force shoes (Leach, 1987), electromyography (Nunamaker and Blauner, 1985; Seeherman *et al.*, 1981), thermography (Purohit and McCoy, 1980; Vaden *et al.*, 1980) and the evaluation of energy expenditure through oxygen consumption (Fedak and Seeherman, 1979).

Although subjective assessment of lameness in domestic animals provides a rapid method of recording the degree of gait abnormality, the value of a reliable, rapid objective assessment method cannot be underestimated (Fackelman and Seeherman, 1983; Leach 1987). Not only would such a system be of diagnostic and prognostic value, but it would allow accurate evaluation of the effects of conservative treatment, surgical intervention and drug therapy. Further, it would provide an opportunity to evaluate fully the reliability and precision of subjective rating scales used for lameness assessment.

For the purpose of clinical measurement, it is important to compare a new measurement technique with an established one to ensure sufficient agreement (Bland and Altman, 1976). Perfect agreement between two methods of measurement would result if the two methods gave exactly the same reading each time a measurement was made. If this were the case, and the results from one of the methods were plotted against the results from the second method, all points would lie along a straight line which is referred to as the line of equality. It is also important to have an indication of measurement errors in both techniques before replacing the old method with the new (Krebs *et al.*, 1985). It was considered that the application of the VAS to the measurement of lameness might increase the degree of sensitivity over the commonly used NRS. Consequently, it was decided to compare the reproducibility, repeatability and sensitivity of a VAS and an NRS when used to score lameness, using sheep as a model. Reproducibility is a measure of between-observer variability, which can be defined as the closeness of agreement between scores obtained by the use of the same scoring system on the same animal by different observers (British Standards Institution, 1979). Repeatability is a measure of within-observer variability, which can be defined as the closeness of agreement between 2 mutually independent score results obtained under conditions when the results are obtained by use of the same scoring system, on the same animal, by the same observer within short time intervals (British Standards Institution, 1979).

The importance of ensuring that the person using a subjective rating scale receives a full explanation of its use, and becomes familiar with the scale prior to assessment is generally recognised (Huskisson, 1974; Seymour, 1982). In order to investigate this finding, an untrained observer was used to score lameness in sheep to compare the reproducibility and repeatability of scores awarded by an untrained observer with those of 2 trained observers.

Experimental models of pain in the domestic species frequently fail to detect the analgesic activity of non-steroidal anti-inflammatory drugs (NSAIDs), which are found to be potent analgesics when used clinically (see chapter 1 and chapter 4). The VAS has been widely used in the field of rheumatology for evaluation of analgesic efficacy (Downie *et al.*, 1978b) and also it is used frequently in clinical trials (Huskisson, 1982; 1983). Footrot (see chapter 1) is an extremely painful naturally occurring condition of the epidermal tissues of the ruminant foot (Egerton, 1989; Stewart, 1989). Consequently, it was decided to investigate the power of the VAS as a method of detecting changes in lameness, induced by footrot, in sheep after administration of a NSAID.

6.2 Materials and Methods

6.2.1 Animals

6.2.1.1 Study 1

Sixty-two adult non-pregnant females were used. They were aged from 2 to 4 years and were of mixed breed. These sheep were selected from the flock by a shepherd as sheep believed to be suffering from forelimb lameness. All were free from clinical signs of systemic disease at the time of the study. They were maintained on grass as a group separate from the main flock. No supplementary feeding was provided and water was available *ad libitum*. These sheep were brought from the field to a holding area 1 hour prior to the onset of the experimental procedure to allow the sheep to settle.

6.2.1.2 Study 2

A total of 25 sheep were used. Sheep numbers 1 to 8 were Scottish Blackface lambs aged approximately 8 to 10 weeks and sheep numbers 9 to 25 were non-pregnant females, also Scottish Blackface, aged from 2 to 4 years. These sheep were selected from a different flock by a shepherd as sheep believed to be suffering from footrot. All were free from clinical signs of systemic disease at the time of the experiment. They were maintained on grass as a group with the main flock. No supplementary feeding was provided and water was available *ad libitum*. These sheep were separated from the main flock and brought to the holding area prior to the onset of the experimental procedure to allow the sheep to settle.

Drug treatment

Flunixin meglumine was administered by intravenous (IV) bolus injection at a dose rate of 1.0 mg per kg to sheep numbers 1 to 6 and at a dose rate of 2.0 mg per kg to sheep numbers 9 to 23. Sheep numbers 7, 8, 24 and 25 received no treatment.

6.2.2 Subjective lameness assessment

Observers

6.2.2.1 Study 1

A total of three observers were used. All 3 observers were veterinary surgeons and were familiar with lameness assessment in domestic animals. Observer 1 and 2 were experienced in scoring sheep lameness specifically and were experienced in the use of the

VAS, both for scoring lameness and also for scoring signs of pain in dogs. Observers 1 and 2 were also experienced in the use of NRS to score lameness. Observer number 3 was unfamiliar with the use of VAS although she was familiar with the NRS method of subjective scoring.

To assess reproducibility (between-observer variability), sheep numbers 1 to 45 were scored for lameness on 1 occasion by observer 1 and also by observer 2. Sheep numbers 1 to 25 were similarly scored for lameness on 1 occasion by observer 3. The third observer scored sheep numbers 1 to 25 at the same time as observers 1 and 2. The observers viewed the sheep at the trot, both to the left and to the right and also when turning. No restriction was placed on the time taken to reach a decision on the degree of lameness shown by an individual sheep, although a score was generally awarded within 3 minutes. The observers did not collaborate over the score awarded to individual sheep although they initially agreed on which fore limb the sheep was lame.

To assess repeatability (within-observer variability), sheep numbers 46 to 62 were scored for lameness on two occasions, one hour apart, by observer 1 and also by observer 2. Observers 1 and 2 did not have access to the first score awarded when scoring sheep numbers 46 to 62 on the second occasion 1 hour later. Sheep numbers 46 to 55 were similarly scored for lameness on 2 occasions by observer 3. The third observer scored sheep numbers 46 to 55 at the same time as observers 1 and 2. Lameness was scored as described (*vide supra*).

6.2.2.2 Study 2

Observers 1 and 2 (*vide supra*) were used. Sheep numbers 1 to 25 were scored for lameness by both observers on 5 occasions. The sheep were initially scored for lameness before drug administration and subsequently at 1, 2, 4 and 6 hours after drug injection. Lameness was scored as described previously. The sheep were assessed for overall lameness rather than lameness on 1 specific limb as frequently more than one limb was affected.

6.2.3 Lameness rating scales

Numerical rating scale

In study 1, an NRS was completed by each observer on each occasion that they scored a sheep for lameness. The NRS consisted of five divisions represented by the numbers 0, 1, 2, 3 and 4. Only four of these divisions characterised lameness. The observers had the following descriptions of each division provided and were able to refer to them as required; 0 = clinically sound/no evidence of lameness; 1 = barely detectable lameness; 2

= obvious lameness; 3 = severe head nod and possibly resting the affected foot when standing; 4 = carrying foot at the trot.

Visual Analogue Scale

In both studies a VAS was completed by each observer on each occasion that lameness was scored. A new score sheet was used for each sheep each time it was assessed for lameness. The VAS was a straight horizontal line, 100 mm long, whose extreme limits were marked with perpendicular lines. Both ends of the scale carried a verbal description. The descriptions used were as follows; 0 mm = clinically sound, 100 mm = could not be more lame (Fig. 6.1). The observer marked the line at a position which represented to them, the severity of lameness exhibited by the sheep under investigation.

6.2.4 Study Design

6.2.4.1 Study 1

Forty-five sheep were selected at random (sheep numbers 1-45) and in order to assess reproducibility (between-observer variability), observers 1 and 2 scored these sheep for lameness using a VAS. In addition, each observer completed an NRS score for the same 45 sheep.

The remaining 17 sheep were scored on 2 occasions, 1 hour apart, to investigate the repeatability (within-observer variability) of both the VAS and the NRS. The sheep were not identified by number (sheep numbers 46-62) to the observers until after the second scoring period was completed.

In order to evaluate how familiarity with use of VAS and NRS could affect the reproducibility and repeatability of the two scales, observer 3 scored sheep numbers 1 to 25 for lameness on 1 occasion using both a VAS and an NRS, and sheep numbers 46 to 55 for lameness on 2 occasions 1 hour apart.

6.2.4.2 Study 2

Twenty-five sheep were scored for lameness by 2 observers using a VAS. The effect of a potent NSAID, flunixin meglumine on the severity of lameness was evaluated. The drug was administered at one of two dose rates. Sheep numbers 1 to 6 (group A), received 1.0 mg/kg, IV, and sheep number 9 to 23 (group B), received 2.0 mg/kg, IV. The remaining 4 sheep, numbers 7, 8, 24 and 25 were not treated. The 2 observers scoring lameness were unaware of the number of sheep which did not receive treatment. They were

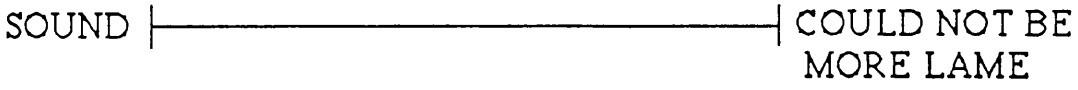

Figure 6.1. Format of the visual analogue scale (VAS) used in the assessment of lameness.

The VAS was a 10 cm line (not drawn to scale) and observers were asked to mark the line at the position which represented the severity of lameness.

informed that one of two different dose rates would be administered. All sheep were scored for lameness on 4 occasions after drug administration at 1, 2, 4, and 6 hours. The observers were not given access to previously recorded scores for individual sheep when scoring post-treatment lameness, and the sheep were not identified by number to the observers.

6.2.5 Statistical analyses

Study 1

The VAS data were expressed to the nearest 0.5 mm, whereas the individual discrete values awarded for the NRS were used in the results. The distributions for scores were investigated for normality, using normal probability plots and correlation analysis. Statistical significance of differences between observers when scoring lameness by the VAS was determined by use of a paired Student's *t* test on the differences obtained between the 2 observers for the same sheep. Differences within observer, when scoring the same sheep on 2 occasions were similarly analyzed. When scoring lameness by the NRS, differences were more appropriately analyzed using the Wilcoxin signed rank test.

Statistical tests were carried out using a 5% significance level.

Study 2

There were four control sheep in this study. These sheep were not included in the statistical evaluation of the study.

The VAS data were expressed to the nearest 0.5 mm. In view of the unbalanced study design, with 6 sheep in group A and 15 sheep in group B, statistical analysis was undertaken using a general linear model routine. The experimental design was suitable for analysis using a 3 and 2 factor nested analyses of variance. The 3 factor nested design provided statistical tests for treatment, observer and time factors and their two-way interactions. The 2 factor design was applied at individual time points and provided tests for differences between treatments, differences between observers and treatment x observer interaction. Animals were regarded as a random factor whereas treatments, observers and time were regarded as fixed factors.

Statistical tests were carried out using a 5% significance level.

6.3 Results

6.3.1 Study 1

6.3.1.1 Reproducibility (between-observer variability)

The VAS and NRS scores assigned by observer 1 and observer 2 to sheep numbers 1 to 45 are shown in Table 6.1.

The distribution of VAS scores obtained for observers 1 and 2 did not indicate normal distributions, and this was confirmed by statistical analysis, using the correlation test for normality. Instead, most VAS values were close to the extremes and a few values were in the middle of the scale. Differences between the scores of the 2 observers for the same sheep had a mean value of -0.46 and a range from -25.5 to 23.5. These differences did appear to have a normal distribution. Five sheep were awarded VAS scores which resulted in a difference between observers of ≥20 mm (sheep numbers 2, 8, 13, 21 and 23). The observers were in full agreement on 7 occasions, and as might be expected, 6 of these 7 sheep were observed to be sound. Observer 1 awarded a higher score than did observer 2 for 18 sheep, and observer 2 awarded a higher score than did observer 1 for 20 sheep. However, for most sheep the 2 observers were in close agreement, and 64% of the scores for the same sheep were within ± 10 mm. Statistical analysis indicated that the differences between the 2 observers when scoring lameness by use of a VAS were not significant.

The distribution of NRS scores obtained for observers 1 and 2 indicated normal distributions, and this was confirmed by statistical analysis, using the correlation test for normality. The distribution of NRS scores for each observer were remarkably similar, each observer mostly recording a score of 0 or 1. The observers awarded the same lameness score to 20 of the sheep and awarded scores that differed by 1 discrete scoring unit to a further 23 sheep. The remaining 2 sheep were awarded scores that differed by 2 units. Six of the sheep scored for lameness were found to be sound by both observers, as was the case when they were scored by VAS. Statistical analysis of the data indicated that the differences between observers, using the NRS, were not significant.

6.3.1.2 Repeatability (within-observer variability)

The results for scoring 17 sheep for lameness using a VAS and an NRS on 2 occasions, 1 hour apart are shown in Table 6.2.

	VAS			NRS		
	Obs 1	Obs 2	Diff	Obs 1	Obs 2	Diff
Sheep1	10.0	11.0	-1	1	1	0
2	12.0	32.0	-20.0	1	2	-1
3	0	0	0	0	0	0
4	4.0	0	4.0	1	0	1
5	0	4.0	-4.0	0	1	-1
6	5.5	0	5.5	1	0	1
7	0	0	0	0	0	0
8	6.5	32.0	-25.5	1	2	-1
9	32.0	48.0	-16.0	2	2	0
10	0	9.0	-9.0	0	1	-1
11	27.5	24.5	3.0	2	2	0
12	11.0	15.5	-4.5	1	1	0
13	23.5	0	23.5	2	0	2
14	7.0	0	7.0	1	0	1
15	0	0	0	0	0	0
16	23.0	11.0	12.0	2	1	1
17	14.0	1.5	12.5	1	1	0
18	3.0	0	3.0	1	0	1
19	12.5	4.5	8.0	1	1	0
20	7.0	0	7.0	1	0	1
21	20.5	0	20.5	2	0	2
22	0	16.0	-16	0	1	-1
23	6.0	30.0	-24.0	1	2	-1
24	0	0	0	0	0	0
25	0	0	0	0	0	0
26	79.0	89.0	-10.0	4	4	0
27	69.0	72.0	-3.0	4	3	1
28	29.0	11.0	18.0	2	1	1
29	45.0	31.0	14.0	3	2	1
30	15.0	16.0	-1.0	2	1	1
31	0	15.0	-15.0	0	1	-1
32	0	0	0	0	0	0
33	4.0	4.0	0	1	1	0
34	17.0	0	17.0	1	0	1
35	7.0	3.0	4.0	1	1	0
36	69.0	88.0	-19.0	3	3	0
37	85.0	98.0	-13.0	3	4	-1
38	67.0	53.0	14.0	3	2	1
39	0	2.0	-2.0	0	1	-1
40	96.0	97.0	-1.0	4	4	0
41	87.0	92.0	-5.0	4	4	0
42	8.0	6.0	2.0	1	1	0
43	0	5.0	-5.0	0	1	-1
44	18.0	19.0	-1.0	1	1	0
45	68.0	65.0	3.0	4	3	1

Table 6.1. Scores assigned by observers 1 and 2 when a group of 45 sheep were rated for lameness, using a continuous scoring system (visual analogue scale; VAS) and a discrete scoring system (numerical rating scale; NRS) on the same occasion.

Differences (Diff) between scores of the observers (obs) are also shown.

	VAS						NRS					
	Obs 1			Obs 2			Obs 1			Obs 2		
	T 1	T 2	Diff	T 1	T 2	Diff	T 1	T 2	Diff	T 1	T 2	Diff
Sheep 46	35.0	0	35.0	33.0	0	33.0	2	0	2	2	0	2
47	26.0	7.0	19.0	0	7.0	-7.0	2	1	1	0	1	-1
48	32.5	22.0	10.5	32.5	22.0	10.5	2	2	0	2	2	0
49	53.0	34.5	18.5	15.0	32.0	-17.0	3	2	1	1	2	-1
50	0	9.0	-9.0	14.0	0	14.0	0	1	-1	1	0	1
51	14.0	15.5	-1.5	0	2.0	-2.0	1	1	0	0	1	-1
52	28.0	10.0	18.0	42.0	27.0	15.0	2	1	1	2	2	0
53	14.0	31.0	-17.0	3.5	4.0	-0.5	1	2	-1	1	1	0
54	0	12.0	-12.0	18.0	5.5	12.5	0	1	-1	1	1	0
55	0	4.5	-4.5	0	0	0	0	1	-1	0	0	0
56	38.0	34.0	4.0	0	23.0	-23.0	2	2	0	0	2	-2
57	0	0	0	0	0	0	0	0	0	0	0	0
58	40.0	34.0	6.0	20.0	31.0	-11.0	2	2	0	2	2	0
59	35.0	46.0	-11.0	56.0	62.0	-6.0	2	2	0	2	3	-1
60	0	0	0	0	6.0	-6.0	0	0	0	0	1	-1
61	54.0	36.0	18.0	5.0	10.0	-5.0	3	2	1	1	1	0
62	57.0	10.0	47.0	30.0	14.0	16.0	3	1	2	2	1	1

Table 6.2. Scores assigned by observers 1 and 2 when a group of 17 sheep were rated for lameness, using the visual analogue scale (VAS) and the numerical rating scale (NRS), on 2 occasions (T1 and T2), 1 hour apart.

The differences (Diff) between the scores of the observers (obs) are also shown.

For time 1 and time 2, the mean VAS scores (± SEM) for observer 1 were 25 (± 5.0) and 18 (± 3.6), respectively. For observer 2 at time 1 and time 2, the mean VAS scores (± SEM) were 15.8 (± 4.2) and 14.4 (± 4.0), respectively. The difference between the scores awarded to each individual sheep on each occasion was also determined. Agreement between the 2 scores should result in a difference of zero. This was only achieved by observer 1 for sheep 57 and 60 and by observer 2 for sheep 55 and 57. Observer 1 judged 4 sheep to be lame on only 1 occasion, whereas observer 2 judged 6 sheep to be lame on only 1 occasion. Both observers scored most of the sheep within 20 mm of the previous score. Statistical analysis of the data indicated that the differences were not significant.

Using the NRS, there were no differences between the scores awarded at the first and second assessment times by observer 1 for sheep 48, 51, 56, 57, 58, 59 and 60 and by observer 2 for sheep 48, 52, 53, 54, 55, 57, 58 and 60. Observer 1 judged 4 sheep to be lame on only 1 occasion, whereas observer 2 judged 6 sheep lame on only 1 occasion. Of sheep not achieving the same score on both occasions, most were scored within 1 discrete unit of their previous score. Statistical analysis of the data indicated that the differences were not significant.

6.3.1.3 Comparison of VAS and NRS

To compare the precision of the VAS and the NRS when used to score lameness, a precise objective measurement of lameness would be required with which to compare the subjective rating scales. No objective measurement of lameness was made during this study, therefore, the VAS and the NRS were evaluated by comparing one with the other directly. The degree of linear correlation (r) between the 2 measurement methods for observers 1 and 2 was 0.94 and 0.95, respectively. Despite a similar degree of correlation between methods for both observers, agreement between the methods was not perfect, because all points did not lie on the theoretical line of equality (Fig. 6.2a and 6.2b). The line of equality is that line on which all points would lie if the VAS and NRS were in perfect agreement. Consequently a line of best-fit was constructed by plotting a curved line through the median VAS values for each of the NRS scores (Fig. 6.2a and 6.2b). For small VAS values, the best-fit line for both observers was convex and was clearly above the theoretical line of equality, whereas for large VAS values, the best-fit line was concave.

For each observer, a score of 1 on the NRS was associated with the range 0 to 20 mm on the VAS, and the values tended to lie above the theoretical line of equality. Similarly, when observer 1 awarded a score of 2 on the NRS, the equivalent VAS value was <40

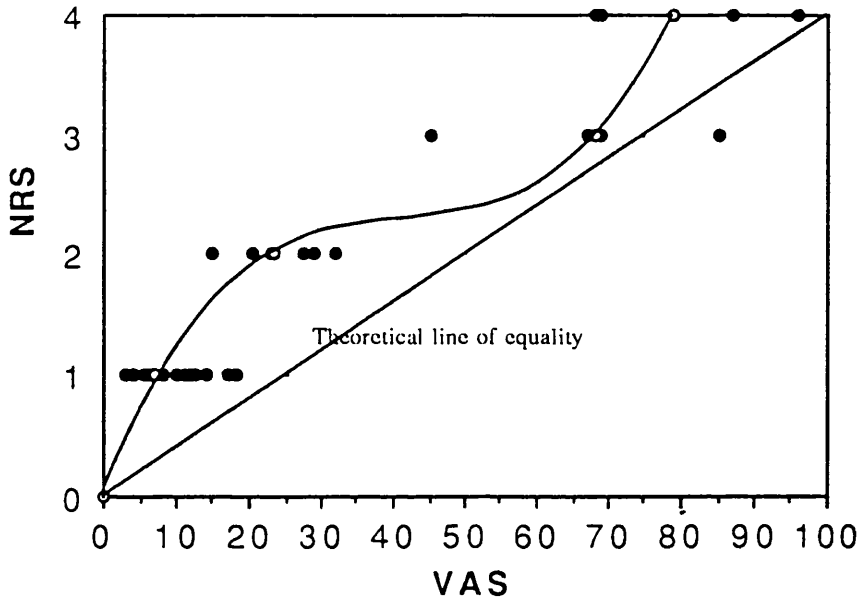

Figure 6.2a . The scores awarded to 45 sheep for lameness by observer 1 using the visual analogue scale (VAS), plotted against the scores awarded to the same 45 sheep using the numerical rating scale (NRS).

The curved line of best-fit is plotted through the median of the VAS values awarded at each NRS score. The theoretical line of equality is shown.

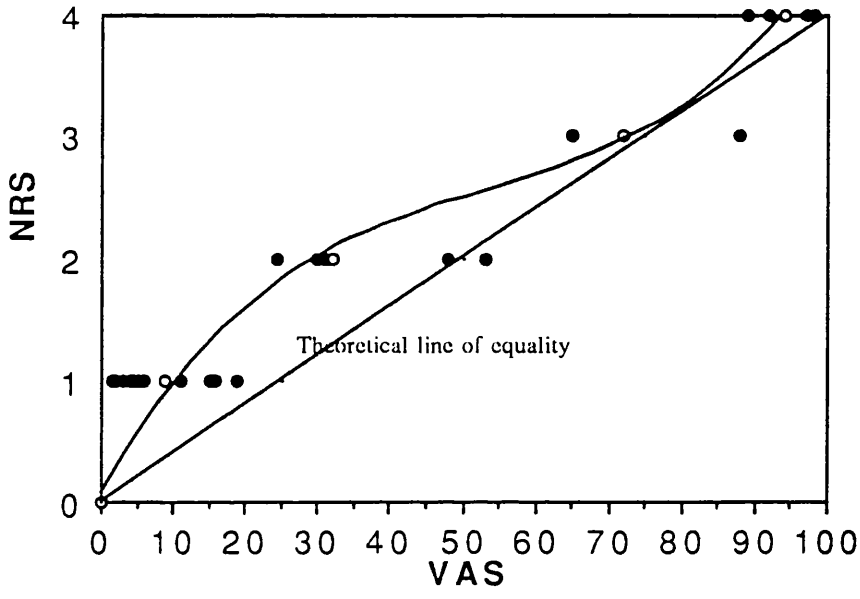

Figure 6.2b . The scores awarded to 45 sheep for lameness by observer 2 using the visual analogue scale (VAS), plotted against the scores awarded to the same 45 sheep using the numerical rating scale (NRS).

The curved line of best-fit is plotted through the median of the VAS values awarded at each NRS score. The theoretical line of equality is shown.

mm. However, observer 2 had a wider range of VAS values, <60 mm associated with the NRS score of 2, and these values crossed the theoretical line of equality. The same trend was seen for sheep awarded an NRS score of 3 by both observers, the VAS values that lie in the range 45 to 85 mm again crossed the theoretical line of equality. Apart from one sheep awarded a score of 45 mm on the VAS with an NRS score of 3 by observer 1, all other sheep awarded an NRS score of 3 by observer 1 or 2, had VAS values >65 and >68 mm, respectively. For both observers, an NRS score of 4 was associated with VAS values >68 and these again tended to lie above the theoretical line of equality. Fewer visual analogue scores were located in the region of 40 to 70 mm by both observers.

6.3.1.4 Influence of learning on the use of subjective rating scales

Reproducibility

The scores for observers 1, 2 and 3 when scoring sheep numbers 1 to 25 for lameness using a VAS and an NRS are shown in Table 6.3. The distribution of VAS scores for observer 3 suggested a normal distribution, and this was confirmed by statistical analysis using the correlation test for normality. The VAS scores awarded by observers 1, 2 and 3 to the same 25 sheep were in full agreement on only 1 occasion when sheep number 24 was observed to be sound by all 3 observers (Table 6.3). Observer 3 awarded a higher VAS score than either observer 1 or observer 2 to 21 of the 25 sheep. Four sheep scored as sound by observers 1 and 2 were judged to be lame by observer 3. Again, for most sheep, observers 1 and 2 were in close agreement, and 64% of the scores for the same sheep were within ± 10 mm. This was the same percentage as shown for the same two observers scoring a total of 45 sheep for lameness. Observer 3 scored only 36% of sheep within ± 10 mm of observer 1 and 40% of sheep within ± 10 mm of observer 2. Statistical analysis indicated that there was a highly significant difference between the VAS values awarded to the same animal by observer 1 and observer 3, $p \leq 0.0001$, and similarly between observer 2 and observer 3, $p \leq 0.001$.

The distribution of NRS scores for observer 3 also suggested a normal distribution, and again this was confirmed by statistical analysis using the correlation test for normality. Observers 1, 2 and 3 only awarded three sheep (1, 17 and 24) the same NRS score. For 10 animals, observer 3 was in agreement with only 1 of the other 2 observers. Statistical analysis indicated that there was a significant difference between the NRS values awarded to the same animal by observer 1 and observer 3, $p \leq 0.01$, and observer 2 and observer 3, $p \leq 0.01$.

	VAS			NRS		
	Obs 1	Obs 2	Obs 3	Obs 1	Obs 2	Obs 3
Sheep No. 1	10.0	11.0	12.0	1	1	1
2	12.0	32.0	24.0	1	2	2
3	0	0	12.0	0	0	1
4	4.0	0	8.0	1	0	1
5	0	4.0	12.5	0	1	1
6	5.5	0	17.0	1	0	2
7	0	0	1.5	0	0	1
8	6.5	32.0	32.5	1	2	2
9	32.0	48.0	63.0	2	2	3
10	0	9.0	0	0	1	0
11	27.5	24.5	61.0	2	2	3
12	11.0	15.5	0	1	1	0
13	23.5	0	37.0	2	0	2
14	7.0	0	8.0	1	0	1
15	0	0	43.0	0	0	2
16	23.0	11.0	43.5	2	1	2
17	14.0	1.5	16.5	1	1	1
18	3.0	0	18.0	1	0	1
19	12.5	4.5	57.0	1	1	2
20	7.0	0	46.0	1	0	2
21	20.5	0	21.5	2	0	1
22	0	16.0	40.0	0	1	2
23	6.0	30.0	49.0	1	2	2
24	0	0	0	0	0	0
25	0	0	5.0	0	0	1

Table 6.3. Scores assigned by 2 observers (Obs), 1 and 2, familiar with the use of a visual analogue rating scale (VAS) and an numerical rating scale (NRS), and 1 untrained observer, observer 3 where 25 sheep were rated for lameness on 1 occasion.

Repeatability

The scores assigned to a group of 10 sheep (sheep numbers 46-55), on two occasions one hour apart, by observers 1, 2 and 3 using both a VAS and an NRS are shown in Table 6.4.

For time 1 and time 2, the mean VAS scores (± SEM) for observers 1, 2 and 3 were 20.3 (± 5.6), 15.8 (± 4.9), 41.0 (± 8.2) and 14.6 (± 5.6), 10.0 (± 3.9) and 39.3 (± 3.9) respectively. Observer 3 awarded scores within 20 mm of the previous score to 50% of sheep. Statistical analysis of the data indicated that the differences between the VAS scores awarded by observer 3 at time 1 and the VAS scores awarded by the same observer at time 2 were not significant.

Observer 3 awarded the same NRS score at each recording interval to five sheep (sheep numbers 1, 2, 4, 7 and 10). Statistical analysis of the data indicated that the differences between the scores awarded at time 1 and the scores awarded at time 2 were not significant.

Comparison of VAS and NRS

The degree of linear correlation between the two scoring methods when used to score 25 sheep on 1 occasion by observer 3 was 0.9. Although there was a good degree of correlation between the two methods, clearly agreement between the methods was not perfect, because all points did not lie on the theoretical line of equality. A line of best-fit was constructed by plotting a curved line through the median VAS values for each of the NRS scores (Fig. 6.3). For small VAS values, the best-fit line was convex and clearly above the theoretical line of equality, whereas for larger VAS values, the best-fit line tended towards concavity, but remained above the theoretical line of equality. A score of 1 on the NRS was associated with the range 5 to 21.5 mm on the VAS. Similarly when observer 3 awarded a score of 2 on the NRS the equivalent VAS value was <57 mm. Observer 3 awarded an NRS score of 3 to only 2 sheep where the equivalent VAS values were 61 and 63. No sheep were awarded a maximum NRS score of 4. Most visual analogue scores were located in the region between 12 and 49 mm.

6.3.2 Study 2

6.3.2.1 Differences between observers

Table 6.5a shows the VAS scores, and the mean and SEM of the scores, awarded by observer 1 and observer 2 to sheep in group A. Table 6.5b shows the VAS scores, and

	Obs 1			Obs 2			Obs 3		
	T 1	T 2	Diff	T 1	T 2	Diff	T 1	T 2	Diff
Sheep 46	35.0	0	35.0	33.0	0	33.0	50.0	47.5	2.5
47	26.0	7.0	19.0	0	7.0	-7.0	47.0	45.0	2.0
48	32.5	22.0	10.5	32.5	22.0	10.5	62.0	20.0	42.0
49	53.0	34.5	18.5	15.0	32.0	-17.0	60.5	56.0	4.5
50	0	9.0	-9.0	14.0	0	14.0	0	45.0	-45.0
51	14.0	15.5	-1.5	0	2.0	-2.0	60.0	22.0	38.0
52	28.0	10.0	18.0	42.0	27.0	15.0	48.0	47.0	1.0
53	14.0	31.0	-17.0	3.5	4.0	-0.5	6.5	43.0	-36.5
54	0	12.0	-12.0	18.0	5.5	12.5	67.5	43.0	24.5
55	0	4.5	-4.5	0	0	0	8.5	24.5	-16.0
	Obs 1			Obs 2			Obs 3		
	T 1	T 2	Diff	T 1	T 2	Diff	T 1	T 2	Diff
Sheep 46	2	0	2	2	0	2	2	2	0
47	2	1	1	0	1	-1	2	2	0
48	2	2	0	2	2	0	3	1	2
49	3	2	1	1	2	-1	3	3	0
50	0	1	-1	1	0	1	0	2	-2
51	1	1	0	0	1	-1	2	1	1
52	2	1	1	2	2	0	2	2	0
53	1	2	-1	1	1	0	1	2	-1
54	0	1	-1	1	1	0	3	2	1
55	0	1	-1	0	0	0	1	1	0

Table 6.4. Scores assigned by observers (obs) 1, 2 and 3 when a group of 10 sheep were rated for lameness, using a visual analogue scale (VAS) and an numerical rating scale (NRS) on 2 occasions (T1 and T2), 1 hour apart.

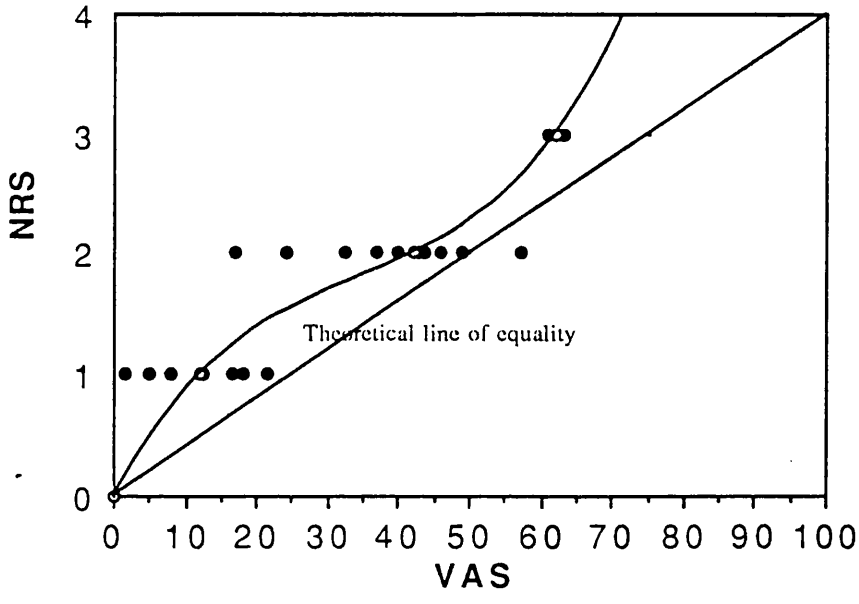

Figure 6.3. The scores awarded to 25 sheep by observer 3 using the visual analogue scale (VAS), plotted against the score awarded to the same 25 sheep using the numerical rating scale (NRS).

The curved line of best-fit is plotted through the median of the VAS values awarded at each NRS score. The theoretical line of equality is shown.

	Obs 1 Sheep No.									
Time(hr)	1	2	3	4	5	6	7	8	Mean	±SEM
0	46.5	30.5	21.0	8.0	89.0	84.0	68.0	79.0	46.5	13.7
1	35.5	18.0	8.0	13.0	60.5	25.0	100	26.5	26.7	10.9
2	7.5	33.0	20.0	0	19.5	20.0	25.0	29.0	16.7	4.5
4	0	46.0	14.0	6.0	35.0	16.0	85.5	67.0	19.5	7.2
6	5.0	17.0	23.5	5.0	21.0	0	78.0	71.5	11.9	4.0
	Obs 2 Sheep No.									
Time(hr)	1	2	3	4	5	6	7	8	Mean	±SEM
0	24.5	22.0	2.5	15.5	83.0	63.0	53.0	77.5	35.1	12.7
1	5.5	13.0	3.0	0	9.5	2.5	58.0	0	5.6	2.0
2	0	14.0	0	6.0	7.0	14.0	47.0	8.5	6.8	2.6
4	0	34.0	0	0	0	0	62.0	38.5	5.7	5.7
6	0	38.5	4.5	1.5	27.5	0	66.0	38.5	12.0	6.8

Table 6.5a. Scores assigned by observers (Obs) 1 and 2 when a group of 8 sheep were rated for lameness using a visual analogue scale before and after the administration of flunixin meglumine, 1.0 mg/kg, IV, at time 0.

The drug was administered at time 0. The mean and standard error of the mean (SEM) of the VAS scores at each time point are shown.

	Obs 1 Sheep No.									
Time (hr)	9	10	11	12	13	14	15	16	17	18
0	34.0	25.0	71.5	100	47.0	24.5	42.0	13.5	100	11
1	32.0	33.5	44.0	100	7.0	13.5	38.0	20.0	62	14
2	34.0	17.5	29.0	100	0.0	14.0	5.5	22.0	75	5
4	18.0	14.5	51.0	100	8.0	9.0	0	31.0	63.5	13
6	0	48.0	45.5	100	3.0	22.0	10.0	34.5	79.5	22
Time (hr)	19	20	21	22	23	24	25	Mean	±SEM	
0	52	43.5	58	41	37.5	46.5	37.0	46.2	10.9	
1	30	48.5	25.5	9.5	49.5	64.0	0	35.1	9.9	
2	11.5	0	18.5	8.5	22	69.0	27.5	24.2	11.4	
4	14	12	44.5	32.5	41.5	50.0	12.0	30.2	10.9	
6	25.5	7.5	18	28	0	56.0	45.0	29.6	11.8	
	Obs 2 Sheep No.									
Time (hr)	9	10	11	12	13	14	15	16	17	18
0	14.0	17.5	93.0	35.0	24.0	12.0	37.5	44.0	70.0	15.5
1	7.0	6.0	20.5	MD	88.0	11.0	13.5	21.0	13.0	3.5
2	5.0	5.0	14.0	94.0	0	15.0	8.5	4.0	31.0	0
4	8.5	70.0	20.0	68.0	7.0	12.5	10.0	22.0	43.0	3.5
6	16.5	14.0	25.0	76.0	9.5	11.5	32.0	22.0	25.0	6.5
Time (hr)	19	20	21	22	23	24	25	Mean	±SEM	
0	28.5	11.0	12.5	5.0	18.5	35.0	19.0	28.7	10.0	
1	8.5	15.0	6.0	0	16.0	29.5	18.0	21.5	8.8	
2	2.0	2.5	9.5	5.0	8.0	25.0	17.0	23.6	9.6	
4	10.5	10.5	19.5	14.0	8.0	41.0	7.0	16.9	6.9	
6	22.0	3.5	13.5	11.0	6.0	28.0	20.0	17.3	7.1	

Table 6.5b. Scores assigned by observers (Obs) 1 and 2 when a group of 17 sheep were rated for lameness using a visual analogue scale (VAS) before and after the administration of flunixin meglumine, 2.0 mg/kg, IV, at time 0.

The drug was administered at time 0. The mean and standard error of the mean (SEM) of the VAS scores at each time point are shown. MD = Missing data.

the mean and SEM of the scores, recorded by observer 1 and observer 2 to sheep in group B. Comparison of the scores awarded by observers 1 and 2 at time 0 *i. e.* prior to drug treatment, to animals in group A and B, indicated that there was a significant difference, $p \leq 0.01$, in the scores awarded to individual sheep at that time. The mean of the VAS scores awarded pre-treatment to group A and B by observer 1 was 46.5 and 46.6 mm respectively, while the mean of the VAS scores for the 2 groups recorded by observer 2 was 35.1 and 28.7 mm respectively. At time 0, and for the remaining time points observer 1 tended to award higher VAS scores to the sheep in both groups than did observer 2. Consequently, the absolute change in VAS scores *i. e.* the difference between pre- and post-treatment scores, was used to examine the between treatment data further.

6.3.2.2 Differences between treatments

Group A

No sheep in group A were found to be sound on initial lameness assessment by either observer (Table 6.5a). However, observer 1 recorded a VAS score of 0 post-treatment for 3 sheep (sheep numbers 1, 4, and 6). Similarly, observer 2 recorded a VAS score of 0 for 6 sheep (sheep numbers 1, 3, 4, 5, 6 and 8) post-treatment.

Table 6.6a shows the percentage decrease in lameness after drug administration recorded by observer 1 and observer 2 for group A. Both observers recorded at least 50% decrease in lameness on 1 or more occasions over the 6 hour post-treatment recording period for sheep numbers 1, 3, 4, 5 and 6. The remaining sheep in group A, sheep number 2, was shown to have at least a 40% decrease in lameness by both observers at 1 hour post-treatment. At 6 hours post-treatment, observer 1 scored 2 sheep more severely lame than at initial assessment (sheep numbers 3 and 7), while observer 2 scored 3 sheep more severely lame than at initial assessment (sheep numbers 2, 3 and 7). All of the remaining sheep were found by both observers to be less lame at 6 hours post-treatment than pre-treatment.

Group B

No sheep in group B were found to be sound on initial lameness assessment by either observer (Table 6.5b). However, observer 1 recorded a VAS score of 0 post-treatment for 6 sheep (sheep numbers 9, 13, 15, 20, 23 and 25). Similarly, observer 2 recorded a VAS score of 0 for 3 sheep (sheep numbers 13, 18 and 22) post-treatment.

	Obs 1 Sheep No.							
Time (hr)	1	2	3	4	5	6	Mean	±SEM
1	23.6	41.0	61.9	-62.9	32.0	70.6	27.8	19.5
2	83.9	-8.2	4.8	100	78.1	76.5	55.9	18.6
4	100	-50.8	33.3	25.0	60.7	81.2	41.6	21.8
6	89.3	44.3	11.9	37.5	76.4	100	59.9	13.9
	Obs 2 Sheep No.							
Time (hr)	1	2	3	4	5	6	Mean	±SEM
1	77.6	40.9	-20.0	100	88.6	96.0	63.9	18.9
2	100	36.4	100	61.3	91.6	77.8	78.9	10.3
4	100	-54.5	100	100	100	100	74.3	25.7
6	100	-75.0	-80.0	90.3	66.9	100	33.7	35.5

Table 6.6a. The decrease in the level of lameness, recorded as a percentage of the pre-treatment visual analogue scores awarded to 6 sheep by observers (Obs) 1 and 2. Flunixin meglumine was administered at 1.0 mg/kg, IV, at time 0.

The mean and standard error of the mean (SEM) for each time point are also shown.

Table 6.6b shows the percentage decrease in lameness after drug administration recorded by observer 1 and observer 2. Observer 1 recorded at least a 50% decrease in lameness for >70% of sheep on 1 or more occasions after drug treatment, and observer 2 found a similar decrease in lameness in >80% of sheep. Six hours after drug administration, 3 sheep, numbers 10, 16 and 18 were assessed as more severely lame than at initial assessment by observer 1. Observer 2 scored 6 different sheep, numbers 2, 3, 9, 12, 21 and 22 more severely lame than at initial assessment at the same time point. All except 1 of the remaining sheep (sheep number 12) were found by both observers to be less lame at 6 hours post-treatment, than pre-treatment. Observer 1 consistently scored sheep number 16 as more severely lame than initially. Observer 1 also scored sheep number 12 at 100 mm on the VAS scale on each occasion. Statistical analysis revealed no significant difference between the 2 treatment groups at any time point post-treatment.

Further statistical analysis of the data revealed that there were no significant interactions between treatments, observers and time.

	Obs 1 Sheep No.								
Time (hr)	9	10	11	12	13	14	15	16	17
1	5.9	-3.4	38.5	0	85.1	44.9	9.5	-48.2	38.0
2	0	30.0	59.4	0	100	42.9	86.9	-63.0	25.0
4	47.1	42.0	28.7	0	83.0	63.3	100	-130	36.5
6	100	-92.0	36.4	0	93.6	10.2	76.2	-156	20.5
Time (hr)	18	19	20	21	22	23	Mean	±SEM	
1	-27.3	42.3	-11.5	56.0	76.8	-32	16.3	10.8	
2	54.6	77.9	100	68.1	79.3	41.3	46.8	11.4	
4	-18.2	73.1	72.4	23.3	20.7	-10.7	28.8	14.4	
6	-100	60.0	82.8	69.0	31.7	100	21.6	20.4	
	Obs 2 Sheep No.								
Time (hr)	9	10	11	12	13	14	15	16	17
1	50	65.7	78.0	MD	-227	8.3	64.0	52.3	81.4
2	64.3	71.4	85.0	-169	100	-25.0	77.3	90.9	55.7
4	39.3	60.0	78.5	-94.3	70.8	-4.2	73.3	50.0	38.6
6	-17.9	20.0	73.1	-117	60.4	4.2	14.7	50.0	64.3
Time (hr)	18	19	20	21	22	23	Mean	±SEM	
1	77.4	70.2	-36.4	52.0	100	13.5	29.3	24.7	
2	100	93.0	22.7	24.0	0	56.8	43.2	18.0	
4	77.4	63.2	4.5	-56.0	-180	56.8	18.5	19.3	
6	58.1	22.8	68.2	-8	-120	67.6	16.0	16.1	

Table 6.6b. The decrease in the level of lameness, recorded as a percentage of the pre-treatment visual analogue scores awarded to 15 sheep by observers (Obs) 1 and 2. Flunixin meglumine was administered at 2.0 mg/kg, IV, at time 0.

The mean and standard error of the mean (SEM) for each time point are also shown.

6.4 Discussion

6.4.1 Study 1

There is a wealth of published data available on gait analysis in horses and dogs, but little has been published on the use or reliability and sensitivity of subjective rating scales such as SDS, GRS, NRS, or VAS in scoring lameness in domestic animals. A subjective response, such as pain intensity, requires an accurate, reliable and sensitive method of measurement (Joyce *et al.* , 1975). The same must be true for other subjective responses, such as lameness. Indeed, in sheep with footrot, signs of pain and lameness are likely to be intimately linked with each other. The early stages of footrot are associated with severe interdigital inflammation, with little or no physical deformation of the foot, and therefore, one must assume that at least initially, the lameness often seen in these animals is a result of the pain experienced when bearing weight.

Precise and objective measurement of lameness in domestic animals, that could be easily used in the clinic or in the experimental situation, would provide an ideal starting point on which to base investigations of the effects of various therapeutic interventions. The precision of other more subjective measurements *e. g.* NRS, VAS, then also could be assessed. Unfortunately, such measurements are not as yet available, although techniques such as electrogoniometry (Adrian *et al.*, 1977; Rowe *et al.*, 1989) may, in time, yield such information.

Normal probability plots of the VAS data for observer 1 and 2 indicated that the population of sheep investigated for lameness were not normally distributed. A similar examination of the NRS data, however, revealed a normal distribution. The 62 sheep in this study were selected from a flock of 300 animals because they were considered by the shepherd to be lame. This group of lame sheep represented <20 % of the entire flock. Within the flock, it can be assumed that most sheep were not markedly lame, and therefore, a normal distribution curve would not be seen. However, we were in effect, looking at a separate population of sheep; they were all specifically chosen because they were believed to be lame. Despite this, we would not expect the data from this group of sheep to have a normal distribution. In any group of sheep with footrot, most will be only mildly lame, with minor interdigital or digital lesions. There will also be a small percentage of sheep with a severe form of the condition, which often will persist chronically (Stewart, 1989), and therefore, a normal distribution curve would not be obtained.

Visual analogue scales provide a continuous method of subjective measurement, and remove the constraints placed on observers by the NRS, which groups information in discrete units. The differences seen in the results may therefore, be explained by virtue of the ability of the VAS to allow more accurate interpretation of the data, because it does not force observers to group unlike data. However, normal probability plots of both the VAS and the NRS data for the 25 sheep scored by observer 3 indicated normal distribution. It is important to note that for this smaller sample of sheep the normal probability plot for the VAS data for both observer 1 and 2 also indicated normal distribution. This may be related to the differences in sample size between the two groups.

If the results for reproducibility, or between-observer variability, of the VAS and the NRS are examined it is clear that both methods, when used for assessment of lameness, provide suitably reproducible results between 2 trained observers. However there were highly significant differences between the untrained observer, observer 3, and the two trained observers when using the VAS and NRS. The untrained observer tended to award sheep a higher score using the VAS than the trained observers, and also recorded 4 sheep lame which both trained observers had scored as sound. Some variation between observers is inevitable, but it is interesting to note that the error in scoring due to visual motor performance alone, is ± 8 mm when using a VAS (Revill *et al.* , 1976). Observers 1 and 2 scored 64% of sheep within ± 10 mm of each other, and it is possible that a portion of this error may be accounted for because of visual motor limitations. However, when the 2 trained observers were compared to the untrained observer, ≤40% of sheep were awarded scores within ± 10 mm, emphasising the differences between the trained and untrained observers.

Dixon and Bird (1981) reported that reproducibility of the VAS can vary along the length of the line, with the greatest reproducibility at the ends of the line and at the centre point. They also indicated that the region ± 20 mm of the centre point of the line causes the most difficulty when allocating scores. It was not possible to test this hypothesis in the present study because of the apparent distribution of lameness present in the study group *i. e.* few sheep were awarded VAS scores in the centre region of the scale (from 30 to 70 mm). However, it is interesting to note that all scores which resulted in differences between observers of ≥20 mm (n=5), involved sheep which were awarded a lameness score of 23 to 32 mm by one observer and 0 to 12 mm by the second observer (Table 6.1). Further, in each case the sheep were awarded a corresponding NRS value of 2 by one of the observers and a value of 1 or 0 by the other observer. To fully evaluate this, a larger group of sheep would be required to ensure that a full range of lamenesses were included within the group.

It is essential to establish that any subjective method used for clinical measurements is repeatable. This allows comparisons to be made, for example, to assess improvement in lameness over a period of days or weeks after treatment has been initiated. Analysis of this data is complex. Within each recording period, each sheep is awarded a subjective score for lameness, and within each recording period this score is assumed not to change. However, it cannot be assumed that the degree of lameness will not alter in the time period between recordings. Because there is no reliable objective method of lameness scoring, there is no way in which any such change can be quantified. Factors that may contribute to alteration in the degree of lameness manifested by sheep include exercise and standing on concrete in the interval between recordings. It has been demonstrated that within-observer comparisons are more sensitive than between-observer comparisons (Maxwell, 1978), and in this study the differences that were seen between measurements made at a one hour interval, were not judged to be statistically significant for either observers 1, 2 or 3 when using either method. Therefore, although observer 3, scored lameness in sheep in a different manner to the trained observers, she demonstrated repeatability using both scoring methods. This indicates that although an observer may be unfamiliar with using an NRS or a VAS for scoring lameness, they would be able to take sequential measurements, and to gauge from these whether or not there had been any improvement or deterioration in lameness.

Perhaps the most important variable to consider when a comparison of the VAS and NRS is made, is the sensitivity of the scoring method. It was not appropriate to evaluate the accuracy of the 2 methods in this study, because that would require a precise measurement of lameness with which the VAS and NRS could be compared. Such a technique in sheep is not available currently. Consequently, the data were examined by evaluating the 2 subjective measurement methods against each other. Correlation measures the strength of relation between 2 variables, and not the agreement between them. The degree of correlation between the VAS and NRS methods was good for all observers. However, assigning significance to these correlation values in this instance, is inappropriate for several reasons. It is the agreement between 2 methods and not the correlation that allows us to assess by how much one method differs from another (Bland and Altman, 1986). Moreover, perfect correlation will exist between two methods of measurement if the points lie along any straight line, whereas perfect agreement is obtainable only if the points lie along the line of equality. The use of 2 interchangeable methods for measuring a clinical variable necessitates close agreement. Finally, any change in the scale of measurement will not affect correlation, but will affect agreement between 2 methods.

The VAS alters the method of measurement from a discrete to a continuous scale and, therefore, can be expected to alter agreement. Perfect agreement between the VAS and NRS, however, cannot be achieved. By design, if a sheep is awarded a score of 0 by 1 method it must also be awarded a score of 0 by the second method. However the same does not apply to the maximal scores. A maximal score (100 mm) by the VAS ('could not be more lame') must correspond to a maximal score of 4 by the NRS, but in this instance, the reverse does not apply as indicated by the results. Sheep may be given a score of 4 (NRS) without achieving a maximal 100 mm score by the VAS. Therefore, the line of equality which is represented on Fig. 6.2a, 6.2b and 6.3, is referred to as the theoretical line of equality. Consequently, use of these scales interchangeably cannot be recommended, because agreement at the upper limits cannot be evaluated. In addition, it can be seen from the data that the 4 lameness categories measured by the NRS do not reflect equal divisions on the VAS. If this were the case, a straight line of best-fit would have resulted.

The verbal descriptions placed at either end of a VAS anchor the scale and should correspond to the absolute maximum and absolute minimum of the parameter being measured (Joyce *et al.*, 1975; Maxwell, 1978). The agreement between the VAS and the NRS could perhaps be altered if the verbal descriptions at the end of the scale were changed. Freyd (1923) stated that the end phrases used should not be so extremely worded as never to be employed. In lameness assessment, one of the extremes is easy to place as an animal can never be less lame than when it is sound on all four limbs. However, the other extreme of the absolute scale is less easy to place. In this study, the statement of 'could not be more lame' was used which might imply that the sheep would score a maximal 100 mm on that scale only if recumbent. A phrase such as 'not touching limb to ground' might be more appropriate because there would be less disagreement between observers as to the meaning of the statement.

It is interesting to note that the line of best-fit for each observer is curvilinear. The point of inflection occurs at approximately 60 and 78 mm for observer 1 and 2, respectively. For observer 1, this correspond to the 'golden section', which is that point on a line which divides it into 2 segments such that the smaller is to the larger as the larger is to the whole line (Benjafield and Adams-Webster, 1976). This point occurs at 62% of the way along the length of a line which in the case of the VAS used in this experiment would be 62 mm. Dixon and Bird (1981) found in an experiment designed to investigate reproducibility along a vertical VAS, that subjects under estimated above the 'golden section' and over estimated below the 'golden section'. If this is true for observer 1 it

would suggest that they tend to over-estimate lameness below 60 and conversely, under-estimate lameness above those values.

Ohnhaus and Adler (1975) indicated that a verbal rating scale produced an artificial augmentation of analgesic drug effect. They concluded that this feature was seen because the data were 'discretised', and that the VAS reflected more precisely what the patient actually felt. Similarly, if a sheep is not completely sound, the observer is obliged to record a NRS score of at least 1 for that animal, although it may, in fact, be perceived to be clinically less lame than the average sheep assigned to that group. It has been demonstrated that both the VAS and the NRS are reproducible and repeatable when used to score lameness in sheep. However, it has been shown that there is not perfect agreement between the VAS and the NRS. Each NRS value had a range of VAS values associated with it which spanned the line of best-fit. This demonstrates an increase in the sensitivity of the VAS when used to score lameness when compared to the NRS. This increase in sensitivity is particularly important at the lower end of a lameness scale where small improvements in lameness may be of great value *e. g.* in detecting the therapeutic effect of a novel analgesic or anti-inflammatory drug.

6.4.2 Study 2

The VAS is a simple, robust, sensitive and reproducible subjective rating scale which is frequently used in clinical trials of analgesic drugs (Huskisson, 1982). In study 1 the VAS was used to assess lameness in sheep, and was found to be simple, reproducible, repeatable and more sensitive than the NRS, with which it was compared. Consequently, it was decided to apply the VAS to the measurement of lameness in sheep which were treated with an analgesic drug to test the power of the VAS as an instrument for the assessment of therapeutic effect.

In order to evaluate the VAS as a tool to detect changes in lameness, it would be preferable to treat the sheep with an analgesic drug with a proven effect in sheep. Unfortunately the drugs which have been demonstrated as effective analgesics in sheep, such as the α_2-adrenoreceptor agonists or the opioids (Livingston *et al.* , 1992b), can result in marked ataxia. Therefore, a NSAID, flunixin meglumine, was chosen to treat the sheep. Flunixin meglumine is a potent NSAID (see chapter 1), and it has been demonstrated as an effective analgesic in laboratory animals (Ciofalo *et al.*, 1975, 1977), humans (Zederfeldt *et al.* , 1977; Sunshine *et al.* , 1984), horses (Houdeshell and Hennessey, 1977; Vernimb and Hennessey, 1977) and more recently in dogs (Reid and Nolan, 1991).

In a study designed to examine the effects of an analgesic drug the tendency of the observers might be to award lower scores post-treatment than pre-treatment with the assumption that an improvement in lameness would occur. To minimise this effect the observers were not allowed access to original lameness scores for sheep. Moreover, when scoring post-treatment lameness they were a) unaware that younger animals had all been assigned to group A and had received only 1.0 mg/kg of flunixin meglumine and b) that an unspecified number of sheep were left untreated. These untreated sheep were not included in the statistical analyses of the data because of the unbalanced design of the study and the small numbers of animals involved.

The 2 observers used in this study were both experienced at using the VAS to record the severity of lameness in sheep. However, statistical analysis indicated that there were significant differences between the scores awarded by observer 1 and the scores awarded by observer 2 pre-treatment. The mean scores for each observer indicated that observer 1 tended to award sheep a higher VAS score than did observer 2, and this remained a constant feature throughout the study period. It is interesting to note that in study 1 there were no significant differences found between the VAS scores awarded by the same 2 observers *i. e.* there was good reproducibility. Prior to the onset of study 1, both observers were scoring sheep for lameness on a regular basis (twice weekly) using the VAS and the NRS. However, at the of time study 2, observer 2 had not scored sheep for lameness using either the VAS or the NRS for approximately 5 months, while observer 1 was using the VAS regularly throughout that period. This indicates that, not only is it important that an observer is familiarised with the VAS prior to the onset of a study, but also that regular use of the system, or a period of retraining using the system is required to maintain a high degree of reproducibility within a study. However, there were no significant observer x time interactions, which confirmed that although the 2 observers were scoring sheep at a different level, they continued to score in a similar manner relative to each other throughout the study period. Therefore, the differences between observers should not have adversely affected the results obtained in study 2. However, to eliminate any differences that did exist due to the way in which the observers recorded lameness, the post-treatment scores were all converted to absolute changes in the VAS score, prior to statistical analysis. This manoeuvre effectively converted the absolute values from the VAS to the difference in lameness intensity values *i. e.* the change in lameness from the original score awarded.

Although there were no significant differences between the drug treatments at any time point after administration of flunixin meglumine, at either dose rate, it was apparent that most sheep in group A (>80%) and group B (>70%) showed a decrease in degree of

lameness after treatment. The sheep included in this study were selected because they were observed to be lame. No animals were excluded because of the degree of severity of lameness and consequently, sheep which were both extremely lame *e. g.* sheep number 12, and sheep that were suffering only a mild degree of lameness *e. g* sheep number 18, were included in the study. Therefore, failure to detect significant changes in lameness post-treatment, may have resulted from the wide spread of values which were recorded from a small number of animals (25) (Quiding and Haggquist, 1983). In order to detect small differences between treatment groups, and scores awarded post-treatment within groups, a larger sample size would have been necessary.

It is possible to obtain an estimate of the number of sheep that should be included in each treatment group to afford the optimum opportunity of detecting a significant change, should one exist, from the data provided by this preliminary study (Lipsey, 1990). The estimate may be derived from a power chart appropriate for a 5% significance level with a power of 80% (Lipsey, 1990). There are 2 pieces of information which are required to operate this chart. Firstly, the order of difference which is sought within the test system must be known. From study 1, when 2 observers score a group of lame sheep on 2 occasions, they award scores within 20 mm of the previous score to most sheep. Therefore, a change of >20 mm could be considered as the most appropriate order of difference to indicate change within the system. Secondly, the normal variation within the population, should be indicated *i. e.* the standard deviation. This information allows the calculation of the effect size value (ES). The ES is calculated by dividing the order of difference required within the system, in this case 20 mm, by the standard deviation of the population of animals which is to be investigated. The power chart then uses this calculated value to estimate the optimal number of animals to include in the study. To detect whether or not there was a significant improvement in the degree of lameness exhibited by the sheep in study 2, approximately 26 sheep would have been required in each treatment group. It is interesting to note, that with the degree of variation within the population of lame sheep which were examined in study 2, in order to detect an improvement of 10 mm on the VAS scale, should one occur, that it would have been necessary to score approximately 130 sheep in each group. Langley and Sheppeard (1984) suggested that when assessing the effect of different drug treatments on pain, the groups used should be matched with care to ensure similar initial pain severity scores, and it has been demonstrated that imbalance in the initial pain severity scores of 2 groups undergoing treatment can affect the outcome of an analgesic trial adversely (Huskisson, 1974). From study 2, it is apparent that it would be appropriate to group sheep, prior to the onset of the study, to ensure similar pre-treatment lameness scores. This would reduce the background variation within the test system thus allowing a reduction in the number of sheep in each treatment group. Moreover, it would allow the introduction of a

more stringent test of improvement without the requirement for a large number of animals *e. g.* an improvement of 10 mm in the VAS lameness score.

The VAS has been used extensively in the field of human rheumatology (Downie *et al.*, 1978b) to evaluate drug efficacy. When assessing the therapeutic effect of an analgesic agent for use in rheumatology, the patients receiving treatment are generally asked to either record their pain relief or the severity of their pain (Langley and Sheppeard, 1984), rather than the clinician assessing the improvement in joint or limb mobility. However a veterinary surgeon prescribing an analgesic in similar circumstances, must rely on the improvement in lameness or joint mobility as an indicator of the analgesic effect of the drug treatment. The VAS used in this study recorded the severity of the lameness exhibited by the sheep at each time point rather than the improvement in lameness from the pre-treatment level. Therefore the VAS used in this study rated absolute values. An absolute VAS utilises the 2 extremes of the condition under investigation at either end of the scale *e. g.* 'sound' and 'could not be more lame'. A comparative VAS however, measures the improvement from the original state recorded *e. g.* one end of the scale might be marked 'no improvement' and the other 'sound'. In this study the observers were rating lameness in sheep, and the sheep were not identified to the observers until after a score had been assigned. Therefore, the observers could not be expected to recall the original score for any particular sheep. One possible solution to such a problem would be to allow the observers access to the original VAS score for each individual sheep. There has been conflicting evidence over the issue of access to previous scores. Several studies found that access to previous scores had deleterious effects on subsequent scores (Jacobsen, 1965; Hart and Huskisson, 1972; Carlsson, 1983) while Huskisson and Scott (1977) argued that although subsequent scores might be influenced by access to previous scores, this led to an increase in precision. The latter authors also suggested that an over-estimation of severity would occur if access was not allowed. In general, the reliability of the comparative scale is poor as it requires recollection of the initial state and further, it does not allow for a deterioration in the condition which can result in bias towards the treatment (Langley and Sheppeard, 1984). However, over a short term, Scott and Huskisson (1979) have shown that patients recording pain can recollect their initial score quite accurately when using a VAS. Consequently, an absolute VAS, measuring severity, probably represents the best choice of linear analogue scale for use in veterinary medicine when assessing lameness.

In conclusion, the VAS and the NRS have been shown to be reproducible and repeatable methods of scoring lameness in sheep, but should not be used interchangeably. Trained observers have greater reproducibility than untrained observers when using both systems, but repeatability remains high. A distinct advantage of the VAS is the improvement in the

degree of sensitivity achieved. In clinical medicine, where the technology for objective measurement frequently is not available, as when measuring lameness, signs of pain or sedation, the use of a reliable and sensitive method of subjective scoring is essential. The VAS provides the opportunity for such a scale to become an integral part of scoring systems. Moreover, from preliminary studies it is apparent that the VAS may provide a useful tool to gauge change in lameness after the administration of therapeutic agents *e. g.* NSAIDs. However it would be important to group animals appropriately in order to ensure a similar initial lameness severity score and to reduce the background variation within the system.

CHAPTER 7

GENERAL DISCUSSION

Current knowledge of the mechanisms and consequences of both acute and chronic pain derive primarily from experimental pain studies, where smaller mammals, including rats , mice and cats, have been used for many years as models for human pain, although occasionally, humans themselves have been employed. In veterinary science, in addition to the smaller mammals, a variety of domestic species, including, dogs, cats and horses have been used as models of experimental pain. Valuable information may be drawn from these studies, not only on the basic mechanisms of pain, analgesia and antinociception which may be applied to mammals in general, but more specifically to the species from which the information was derived. There are a wide range of techniques used to evoke experimental pain, and to assess analgesia and antinociception in humans and animals, but in the domestic species radiant and conducted heat, mechanically-induced pain and balloon models of visceral pain are used most commonly. Recently, sheep were introduced as a model for experimental pain (Nolan *et al.* , 1987a; Livingston *et al.*, 1992a), and these animals have proved useful models for the evaluation of the analgesic effects of the opioids and α_2-adrenoreceptor agonists. In addition, hyperalgesia to mechanical stimulation has been reported in sheep suffering from the naturally occurring infectious disease, footrot, thus these animals may be used as a model of chronic pain, in which the effects of various analgesic agents may be evaluated (Ley *et al.*, 1989; 1990b; 1991a). Importantly, using sheep as an experimental model of pain has encouraged research into the mechanisms of pain and its control in this species, and in food-producing animals generally, a much neglected area. The requirement for effective control of pain in this group of animals is beyond doubt, but unfortunately, this requirement often is counterbalanced by the economic constraints imposed when an unlicensed drug is administered to food-producing animals, rendering the carcase unmarketable.

There is much subjective anecdotal clinical evidence, and little objective experimental evidence, suggesting marked improvement in acute pain in domestic species after administration of certain NSAIDs, including flunixin meglumine and carprofen. To date, failure to demonstrate the antinociceptive effects of the NSAIDs experimentally in the domestic species, probably has been related to the models in which these drugs have been assessed. Generally, to detect an antinociceptive effect of a NSAID, it is accepted that an inflammatory focus, which tends to reduce thresholds to noxious stimulation, must be present. Therefore, attempting to assess the antinociceptive effects of these agents in such models as the balloon model of visceral pain are unlikely to succeed, and consequently, failure to detect an antinociceptive effect of flunixin and carprofen using thermally- and mechanically-induced physiological pain in sheep was not unexpected. It was hypothesized that it would be possible to assess the antinociceptive effects of the NSAIDs

in sheep suffering from the inflammatory condition of footrot. Unfortunately, failure to detect mechanical hyperalgesia in these sheep prevented such an investigation.

Laboratory animals frequently are acclimatized to the environment in which experiments are to be carried out, in order to reduce the stress associated with removing the animal from its companions and familiar surroundings on the day of the experiment. Similarly, because sheep are highly social animals and prone to isolation stress, it has been recommended that they are maintained with at least one other companion during experimental procedures (Livingston *et al.* , 1992a). Despite such precautions, and assessment of control thresholds for each sheep over a period of time, it was found that sheep unaccustomed to the experimental procedures used in these studies had significantly higher thresholds to noxious mechanical stimulation than sheep familiar with experimental procedures. However, repeated exposure to threshold testing over a period of 3 days reduced thresholds to a level comparable to experimental sheep. This raises the possibility that had sheep suffering from footrot been familiarized with testing procedures for a longer period, mechanical hyperalgesia may have been detected. However, it must be questioned whether or not it would be ethical to handle and test sheep with this condition over a period of days without initiating treatment, which may affect the outcome of the experiment. Conversely, it has been reported that mechanical hyperalgesia was detected in sheep which were severely affected with footrot, after clinical resolution of the problem (Ley *et al.* , 1989). Thus, it might be possible to 'train' sheep with footrot to the testing procedure, and also initiate treatment, then subsequently assess thresholds to noxious mechanical hyperalgesia. However, in contrast to normal sheep, thresholds to noxious mechanical stimulation in sheep suffering from footrot, and unaccustomed to experimental threshold testing, did not fall over a period of 3 days, but remained significantly different from experimental sheep. It would be of interest to investigate the mechanisms which caused this finding, although these sheep may simply require a longer period of acclimatization, than normal sheep. Sheep in these studies were selected at random, which should limit the effects of individual variation, however, they were not matched for severity of footrot,which is difficult to achieve, and this may have affected the outcome. Moreover, it would be of interest to monitor changes in plasma levels of 'stress hormones' in sheep with footrot, compared with control animals, during the period of habituation.

Repeat injection of flunixin over the period of acclimatization, caused sheep suffering from footrot to behave in a similar manner to normal sheep *i. e.* after 3 days, mean thresholds to noxious mechanical stimulation, in these sheep, were not significantly different to experimental sheep. It was suggested that the anti-inflammatory and / or the antinociceptive effects of the drug over 3 days, reduced pain in the contralateral forelimb,

frequently also affected with footrot, thus reducing reluctance to bear weight on that limb. If this were the case, it should have been possible to detect an improvement in the degree of lameness over the period of 4 days on which the sheep were tested. Unfortunately, although an assessment of lameness was made on the first day, this was not carried out subsequently. However, the effect of flunixin meglumine on lameness in sheep was evaluated in a later study, and although injection of the drug did not cause a significant improvement in lameness in a 6 hour period after administration, this was probably caused by the experimental design, rather than a lack of efficacy of the drug. Closer matching of sheep in the latter study for level of lameness, or alternatively, including a larger number of sheep in the study, may have allowed more significant conclusions to have been drawn. However, because of the diversity of the pathological changes which occur with footrot, and frequently, the lack of correlation with clinically evident lameness, close matching of groups is difficult.

Rating scales are frequently used to evaluate the antinociceptive activity of the NSAIDs, and other analgesics, in human clinical trials. Such scales should be easy to use and understand, allow accurate interpretation of data, be reproducible and repeatable and perhaps of greatest importance, the scales should be sensitive enough to allow small changes in the test system to be detected (Huskisson, 1974). It is difficult to categorise subjective phenomena, such as pain and relief of pain, distress, anxiety and lameness into a discrete number of equal units, which is a criticism of a number of subjective rating scales currently in use. However, the visual analogue scale (VAS) is a popular tool for assessment of pain and related problems in man, and is gaining favour in veterinary medicine. The VAS has been described for assessment of signs of pain in animals and has been shown to be a reproducible, repeatable and sensitive measure of lameness in sheep. The VAS is regarded as a unidimensional scale and it may be argued that lameness, in a similar manner to pain, should not be considered to be unidimensional. Multidimensional scales such as the McGill Pain Questionnaire (Melzack, 1975), constructed from words used by patients to describe their pain, considers both sensory, affective and evaluative components of pain, and fulfils the criteria for rating scales mentioned above. A similar system for evaluation of animal pain or lameness could be used, however, subjective evaluation of an animal's condition relies on observation of signs, or registration of symptoms, by an observer. Consequently, when evaluating lameness factors including clinical lameness, pain on manipulation, gross pathological change and radiographic changes could be considered. However, it is important to consider the situation in which the scale is to be used, and in many cases in clinical veterinary practice, a scale which is rapid and easy to use would be of primary importance. Presently, the numerical rating scale, verbal rating scale and VAS match this

need and all are reproducible and repeatable. However, the VAS has been shown to provide an in increase sensitivity for no extra time costs.

In 1957, Beecher commented that when evaluating the effectiveness of analgesic agents, the results of studies using experimental pain often were not comparable with those obtained in the clinical situation, as mentioned above for the NSAIDs. To overcome some of the limitations of experimentally-induced pain, he suggested using a tourniquet to induce pain of longer duration, and which in addition produced many of the sympathetic responses associated with pathological pain. These features generally are not associated with physiological pain, such as that produced by noxious mechanical and thermal stimuli in normal animals. Tourniquet-induced pain has been used successfully in man to investigate the analgesic properties of various drugs and reperfusion hyperalgesia has been investigated in rats. In sheep application of an inflated limb tourniquet produced signs of aversion after approximately 20 minutes. Previously, it had been shown that application of a tail tourniquet in rats produced signs of aversion at approximately 12.5 (± 0.2) minutes (Gelgor *et al.* , 1986a), while it was reported that the pain tolerance threshold was reached after 36.2 (± 11.3) minutes in a volunteer study of tourniquet pain in humans (Benzon *et al.* , 1988). Unfortunately, the differences between these studies preclude accurate comparison. Limb tourniquets were used in sheep and humans, whereas a tail tourniquet was used in rats. Moreover, in humans the limb was exsanguinated prior to testing, and this was not carried out in sheep or rats. In human studies of tourniquet pain, the subject is asked to perform certain muscular tasks during ischaemia, and the amount of work done can influence the time to maximum pain. Although the sheep used in the studies of tourniquet pain were restrained in a small pen, it was impossible to regulate the number of times an individual sheep would pace back and forth or indeed lift the limb on which the tourniquet was placed. This must have introduced a degree of error into the subsequent recordings, and may explain why not all sheep underwent the same reduction in thresholds to noxious mechanical stimulation on each occasion. However, tourniquet-induced pain, or hyperalgesia, afforded detection of NSAID antinociception, and also opioid analgesia. Moreover, although application of a tourniquet is undoubtedly a more noxious procedure than inducing physiological pain alone, the sheep retained a degree of control over the duration of the applied stimulus. It would be interesting in the future to correlate physiological changes with signs of aversion in sheep, which perhaps would allow refinement of the method by which aversion is assessed, thus improving the experimental procedure.

Thus, it becomes more obvious that some degree of inflammatory change, or at least release of biochemical mediators of inflammation, is required in order to detect the antinociceptive effects of the NSAIDs. Injection of the mild irritant carrageenan has been

used for many years to evaluate not only the antinociceptive properties of the NSAIDs, but also the anti-inflammatory, or anti-oedema, effects. Intradermal injection of a very low percentage of carrageenan in sheep caused a thermal hyperalgesia of approximately 180 minutes duration, which was reversed by injection of flunixin and carprofen. Curiously, over a period of weeks the response to injection of the irritant in sheep was either lost, or at least limited. This was very surprising, because it appeared that peripheral injection of carrageenan evoked central changes, and such changes are considered usually to persist for some time after the original insult (Woolf, 1989). This finding may have been related to the very low percentage of carrageenan used in the study, 0.0625 %, and it would be of interest to assess the severity of this stimulus in sheep. Injection of carrageenan, 1 %, has been shown to induce oedema but not hyperalgesia in rats, and it was reported that a rise in plasma corticosterone accounted for this finding (Hawörth *et al.*, 1989). Increasing the concentration of the irritant by 100 %, also caused an increase in plasma glucocorticoid levels, but hyperalgesia was detected. Thus, evaluation of plasma levels of the 'stress hormones', such as cortisol, may provide such insight.

It is difficult to achieve a perfect balance between ideal experimental conditions and animal welfare. The provision of accurate controls and well matched treatment groups, 2 of the main benefits of using experimental animals, frequently is difficult to achieve in the clinical situation. However, matching the experimental procedure with the situation of an animal which is experiencing acute or chronic clinical pain, may be fraught with inaccuracies, because of differences between the affective components of the pain experience as well as other factors. Clinically healthy sheep of the same sex, breed and age group were anaesthetised with thiopentone and halothane, underwent ventral midline laparotomy, and were used in an investigation of the pain response of sheep to an acute surgical stimulus. This was achieved by assessing thresholds to noxious mechanical and thermal stimulation both pre- and post-operatively. It was shown that such animals developed a significant thermal hyperalgesia in the post-operative period and that intra-operative administration of flunixin and carprofen, NSAIDs, and buprenorphine, a partial opioid agonist, prevented the development of post-operative hyperalgesia. Changes detected in a pain test system, such as that described, are easier to qualify if a return to control thresholds is demonstrated. Unfortunately, thresholds to noxious stimulation were evaluated for only 2 hours after the end of anaesthesia in sheep, and consequently, the time course of the observed hyperalgesia was not determined accurately. In addition, the duration of drug effect could not be determined. All drugs were administered to sheep after the onset of surgery, and although this did not prevent the detection of an antinociceptive effect of the drugs, it would be of interest to compare the extent and duration of antinociception or analgesia achieved if the drugs were injected pre-

operatively. Although many experimental studies have investigated the neuroanatomical, neurophysiological and neuropharmacological consequences of acute injury, only a limited number of clinical studies have been conducted (Dahl and Kehlet, 1993), and currently more work is required in this area.

The pharmacokinetics of flunixin and carprofen were investigated in a group of healthy sheep, but no correlation between plasma levels of either agent and antinociceptive effect could be made, because changes in thresholds to noxious mechanical or thermal stimulation were not detected in healthy animals. To compare the relative potencies of the agents used in this series of experiments, a wider range of dose rates should be investigated, and it would be of interest to try and correlate drug effect with the level of drug in plasma. After injection of NSAIDs, inhibition of serum thromboxane production is used frequently as an indicator of drug effect. However, not all NSAIDs are potent inhibitors of the enzyme cyclooxygenase, which is required for the production of thromboxane, limiting the value of this procedure in some situations, and measurement of prostaglandin production would face the same problem. Using test systems in which the antinociceptive effect of the NSAIDs can be detected, it should be possible to correlate this effect with plasma drug concentration, regardless of the potency of cyclooxygenase inhibition. However, it has been suggested that it may be more appropriate, in the case of the NSAIDs, to correlate drug effect with the concentration of the NSAID in inflammatory exudate (Lees, 1992). Intradermal injection of carrageenan has been shown to cause thermal hyperalgesia in sheep, and therefore it would be interesting to investigate whether injection of this irritant into a tissue cage (Higgins *et al.* , 1984) would provoke the same response. If so, correlation of NSAID effect, and local concentration of the drug in the tissue cage could be established. However, if the NSAIDs exert central antinociceptive actions, there may be little value in establishing such a relationship.

In addition to the central and peripheral sites of action which have been proposed for the NSAIDs, some workers have claimed a direct antagonistic action at the level of the prostaglandin receptors (Collier and Sweatman, 1968; Rees *et al.* , 1988; Lopez-Bernal *et al.* , 1990), and it was hypothesized that flunixin may exert a similar effect. Preliminary studies in the rat kidney have indicated that this drug does not displace PGE_2 from its binding sites within the kidney, and the effects of flunixin on prostaglandin binding in the spinal cord are being investigated presently.

In conclusion, the antinociceptive effects of the NSAIDs, classically considered for the control of mild to moderate severity, chronic pain, may have been undervalued. It is apparent that pharmacological modulation of the arachidonic acid cascade, is an important method by which acute pain may be attenuated in humans (Dahl and Kehlet, 1991) and

animals (Reid and Nolan, 1991). However, although the role of the NSAIDs as inhibitors of the metabolism of arachidonic acid has been researched widely, and undoubtedly explain many of the anti-inflammatory and anti-pyretic actions of this group of drugs, many avenues remain to be explored before the puzzle of NSAID-induced antinociception can be answered in full. Recent laboratory studies in rats have presented a strong case in favour of a central antinociceptive action of the NSAIDs (Malmberg and Yaksh, 1992a; 1992b), which has been supported by limited clinical evidence in humans. Direct spinal administration of the NSAIDs in sheep using either appropriate experimental models, or clinically in animals undergoing surgery, would provide further valuable information on the mode of antinociceptive action of these agents in farm and other animals.

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