STUDIES ON KERATAN SULPHATE IN EQUINE SYNOVIAL FLUID AND SERUM

Aoibhinn Mc Donnell MVB MRCVS

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Department of Veterinary Surgery, University of Glasgow Veterinary School and Department of Pathological Biochemistry, Glasgow Royal Infirmary, University of Glasgow, 1993

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

Joint disease, particularly osteoarthritis, is a major cause of lameness and loss of athletic performance in the equine industry. Much interest therefore, has focussed on the use of biochemical markers, such as keratan sulphate, a proteoglycan of articular cartilage, as a means of evaluating cartilage and diagnosing osteoarthritis.

In the current study, keratan sulphate was measured using an ELISA (used in human medicine) which was validated for the horse. The assay results were found to be reproducible from day to day and the assay itself was technically convenient to carry out. There was evidence of non-parallelism between the standard curve and equine serum samples, however, this did not make a significant difference to the concentrations of keratan sulphate measured. Synovial fluid and serum levels of keratan sulphate were evaluated in a group of eleven normal horses. There was no correlation between serum and synovial fluid keratan sulphate levels in these animals. There was no difference in synovial fluid keratan sulphate levels measured in paired carpal and tarsal joints in all eleven animals. There was a consistently greater level of keratan sulphate in the left radiocarpal joint compared to the left midcarpal/carpometacarpal joint in all horses, however this data may have been biased due to the relatively small sample number. There was no evidence of day to day or diurnal variation in a group of normal ponies used in the current study, suggesting that a single measurement of keratan sulphate represents a steady state. In a group of six racehorses in training, sampled before and after a period of acute (60 min) exercise, there was a significant elevation in serum keratan sulphate levels, however, this finding was not repeatable on a second test. As only four of the original six horses were available for sampling, the small sample number may have influenced the results obtained on this second test. Serum keratan sulphate levels were evaluated in three horses with osteoarthritis that were treated surgically. There was an elevation in serum keratan sulphate levels two days post-operatively in response to surgical intervention. This finding is in agreement with the results of other authors. However, it was only possible to monitor two of these horses for four days post-operatively, by which time, serum keratan sulphate levels were starting to decrease. In one of these cases, in which serum keratan sulphate was measured for 106 days post-operatively, levels remained consistent and were lower than the values recorded pre and intra-operatively. They did not appear, in this one animal, to be affected by a return to exercise (uncontrolled) and resumption of training. It was concluded that longitudinal sampling to evaluate serum keratan sulphate or synovial fluid keratan sulphate is likely to be of greater value in evaluating articular cartilage status than one-off measurements. Used in conjunction with other tests/biochemical markers, it may provide some useful information on articular cartilage metabolism *in vivo*.

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DECLARATION

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CHAPTER I

INTRODUCTION

Orthopaedic conditions have been shown to be the major cause (68 per cent) of loss of racing performance in horses in the British racing industry (Rossdale, Hopes, Wingfield-Digby and Offord 1985). These authors found that 28 per cent of lameness was attributed to joint disease.

Following appropriate conservative or surgical treatment for acute joint injury due to trauma, or following certain forms of osteochondrosis, many horses achieve complete functional recovery. However, a proportion of these animals go on to develop secondary arthrosis, i.e. degenerative joint disease (DJD), also referred to as osteoarthritis (OA). This condition has been shown to be a major cause of loss of use in athletic performance horses (McIlwraith and Vachon 1988).

OA is ubiquitous in current and past animal and human populations. It is the most common joint disease of man and a major cause of economic loss due to disability (Brandt 1991). Evidence of OA can be found on radiographs of 70 to 80 per cent of people over the age of 55 years, (Brandt 1991), although many patients are asymptomatic (Lane, Michel, Bjorkengren, Oehlert, Shi, Bloch and Fries 1993). The relative age of equine patients (2 years old to 18 years old, Todhunter and Lust 1992) is younger compared to humans. The expectations of therapy are greater in equine patients compared to humans, with complete restoration of normal joint function ultimately demanded for the equine athlete.

It is currently unknown at which stage articular cartilage damage becomes irreversible. Articular cartilage, once damaged, has a very poor capacity for intrinsic repair. It is the key to the functional integrity of the joint. Much interest, therefore, has focused on the biology of this tissue in health and disease and the development of methods to diagnose and monitor the response of articular cartilage to injury as early as possible in the course of disease.

1. ARTICULAR CARTILAGE

1.1 Biology of articular cartilage

Articular cartilage is a dynamic biologic tissue. The major organic components of articular cartilage are Type 2 collagen (85 to 90 per cent of the total) (Mayne and Irwin 1986) and large aggregating proteoglycans (PG) (aggregan). Proteoglycans are enmeshed at a high concentration in a dense network of collagen fibres (Thonar, Bjornsson and Kuettner 1986a). The Type 2 collagen fibrillar network is essential for maintaining the tissue's volume and shape. It gives articular cartilage its tensile strength, a property enhanced by the presence of cross-links, mostly of the pyridinoline class (Eyre, Dickson and Van Ness 1988).

The physiological function of articular cartilage is to resist compression and aid in the dissipation of forces applied to the joint during weightbearing and movement. The high concentration of PGs within the cartilage helps to maintain the collagen network under tension, allowing the tissue to adapt to changes in load and weightbearing. The elastic properties of normal joint cartilage thus result from interactions between the PGs and the collagen network.

Cartilage owes its resistance to compression to the electrostatic repulsion of its thousands of adjacent negatively-charged sulphate and carboxyl groups on the PG molecules. The spaces between the chains of the PG molecules are filled with water, the protons of which are attracted to the negatively charged sulphate groups of the chondroitin sulphate and keratan sulphate chains of the PGs. Approximately 80 per cent of the total weight of normal mature articular cartilage is water, with chondrocytes forming only one to two per cent of the tissue volume (Todhunter and Lust 1992).

1.2. Biology of Proteoglycans

Proteoglycans, the second of the 2 major organic components of the joint, are the key to the integrity of the articular cartilage. PGs are a diverse group of heterogenous complex macromolecular glycoconjugates. They exist as multiple populations with considerable polydispersity (Thonar and others 1986a). They are defined as proteins that contain one or more covalently attached glycosaminoglycan (GAG) sidechains (Kuettner, Aydelotte and Thonar 1991).

Their selective distribution in various anatomic sites, the diversity of their structure and the changes they undergo with age suggest they have specialised functions within the cartilage. These variations in the distribution of collagen and PGs within the articular cartilage may be genetically determined and appear to be characteristic of each individual cartilage (Muir 1978).

The predominant PG in articular cartilage is aggregan, the large aggregating PG which constitutes approximately 80 per cent of the total PG (Lohmander 1988). Aggregan may help to protect PGs from proteolytic degradation (Heinegard and Hascall 1974) or it may function to inhibit cartilage calcification. The remaining PGs are associated with collagen and appear to give cartilage its mechanical stability (Brandt 1981). Dermatan sulphate PGs are also present in articular cartilage and occur as two species of different molecular weight (Rosenberg, Choi, Tang, Johnson, Pal, Webber, Reiner and Poole 1985). As compared with PGs specific to cartilage, monomers of dermatan sulphate are much smaller and do not bind to hyaluronan to form large stable aggregates. Instead they self-associate to form relatively small, unstable aggregates (Rosenberg and others 1985). The large cartilage PG monomer (aggregan) contains typically some 80 to 100 chondroitin sulphate (CS) chains, 30 keratan sulphate (KS) chains, 50 O-linked oligosaccharides and 5 to 10 N-linked oligosaccharides. Each KS chain consists of a linear repeat of disaccharides of N-acetylglucosamine and galactose, (Figure 1) where the hexosamine and sometimes also the galactose, carries a sulphate in the 6-position.

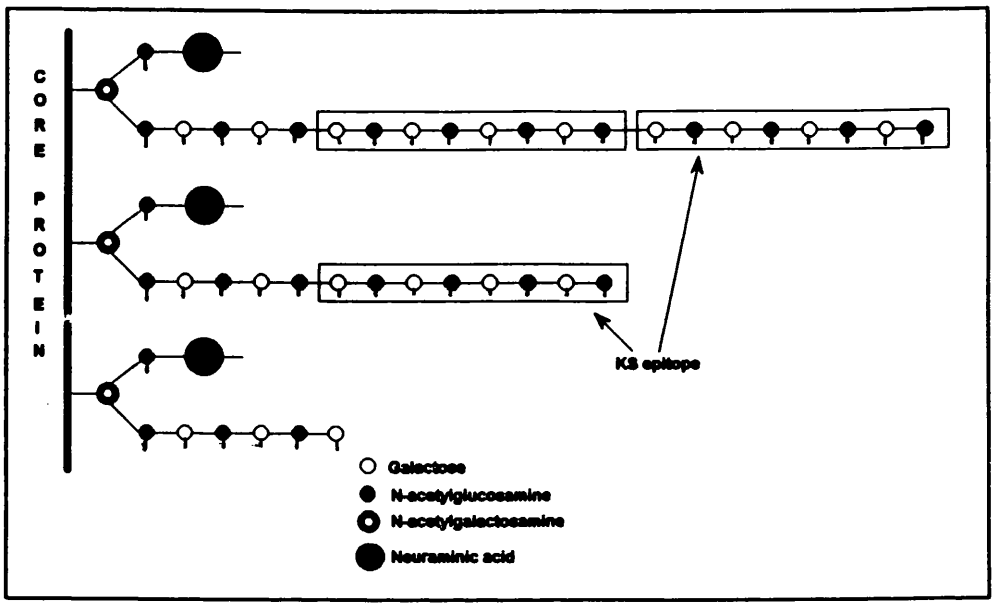

Figure 1

Aggregan with tentative structure of antigenic and nonantigenic KS chains. The longest KS chain shown here contains two contiguous epitopes and can bind at least two antibody molecules. Negatively charged sulphate groups on galactose and N-acetlyglucosamine are shown as short vertical lines.

The linkage of KS to protein is via a glycosidic bond between serine/threonine and N-acetylgalactosamine (Hopwood and Robinson 1974). Following synthesis of the core protein in the endoplasmic reticulum of the chondrocyte and addition of GAG chains in the Golgi (Kimura, Thonar and Hascall 1981), the monomeric aggregating PGs are secreted into the extracellular matrix where they become incorporated into aggregates by binding specifically and non-covalently via their hyaluronate binding (HB) region with a small segment of the hyaluronan molecule (Kimura, Hardingham, Hascall and Solursh 1979). A third protein, link protein, also synthesised by the chondrocyte stabilises the attachment of each monomer to hyaluronan by interacting with both hyaluronan and the HB region of the core protein (Kimura and others 1979). The individual aggregates contain about 40 PG monomers.

The core protein contains three distinct globular domains, (G1 to G3) (Hardingham, Beardmore-Gray, Dunham and Ratcliffe 1986). Most GAG chains are clustered between G2 and G3. The largest region, the CS rich region, contains more than 50 per cent of the core protein and almost all the CS. The majority of the KS chains are located in the intermediate region of the core protein. These chains vary in molecular weight from 2000 to 8000. There are approximately 40 KS chains per molecule (Lohmander 1988).

1.3 Biology of keratan sulphate

More than 95 per cent of the mass of KS in the body is located in hyaline cartilage (including the intervertebral disc) with the balance in cornea, bone and other tissues (Thonar, Williams, Sweet, Maldonado, Lenz, Schnitzer and Kuettner 1988a). Two types of KS occur in mammals, corneal KS (Type 1) and skeletal KS (Type 2). They differ from each other in their average degree of sulphation (Type 2 is more sulphated than Type 1) and in their linkage to protein (N- and 0- linked respectively) (Caterson, Brooks, Sattsangi, Ratcliffe, Hardingham and Muir 1989).

Aggregating PGs of human articular cartilage are relatively rich in KS (Roughley and White 1980). This is true of other vertebrate species in general except PGs in mice and rats which do not appear to contain significant amounts of KS (Venn and Mason 1985). The concentration of KS varies from cartilage to cartilage and also within an area of cartilage (Thonar and others 1986a). PGs containing KS are present primarily in the more basal zones of articular cartilage (Aydelotte and Kuettner 1988). KS is a major component of mineralising calf bone (Meyer 1956), but not of mature bone (Scott and Haigh 1985). It is present in arterial wall (Baker 1989) and in the GAG rich pressure pads of flexor tendons in small amounts (Vogel and Thonar 1988).

Keratan sulphates are typically heterogenous in size and charge, properties that have hindered isolation and characterisation of the KS molecule by standard biochemical techniques. Identification of the structure of KS has been facilitated by the development of KS specific endoglycosidases (Baker 1989).

The contribution of KS chains to the functional properties of the aggregating PGs of articular cartilage is unclear (Thonar and others 1986a). It is possible that the KS chains and related O-linked oligosaccharides offer some protection from proteolytic degradation of the core protein by extracellular proteases secreted by the chondrocytes. Although KS and to a lesser extent the O-linked oligosaccharides contribute to the functional properties of the PG, i.e. swelling pressure and resistance to deformation, this varies with age, tissue and species. KS content increases with age (Thonar and others 1986a) and is often higher in maximally loaded areas.

Sulphated KS may not be an essential ingredient for normal cartilage function, (Thonar, Meyer, Dennis, Lenz, Maldonado, Hassell, Hewitt, Stark, Stock, Kuettner and Klintworth 1986b). Most patients with Type 1 Macular Corneal Dystrophy, a disease caused by precipitation of unsulphated KS-bearing PG in the cornea, do not have normally sulphated KS in their circulation (Thonar and others 1986b). As patients with this condition do not have an increased incidence of cartilage disease, it is likely that the presence of sulphate groups on the keratan chains is not essential for the functional properties of cartilage PG (Thonar, Williams, Sweet, Maldonado, Lenz, Schnitzer and Kuettner 1989a). KS chains of normal length are absent from cartilages of rats and mice (Venn and Mason 1985). Therefore sulphated KS chains provide little advantage even in cartilages subjected to very high loading forces.

2. METABOLISM OF ARTICULAR CARTILAGE

2.1 Metabolism of proteoglycans

Proteinases synthesised by the chondrocytes in inactive form are activated in the matrix and can cleave the core protein of individual aggregating molecules at one or more sites, but usually near the HB region (Figure 2). The fragments containing GAGs then rapidly diffuse out of the matrix into adjacent body fluids. The matrix of adult human cartilage becomes enriched with non-functional PG fragments consisting of a HB region to which few or no GAG chains are attached (Roughley, Poole, Campbell and Mort 1986; Bayliss, Holmes and Muir 1989). These GAG depleted degradation products represent up to 50 per cent of the PG molecules in the matrix (Bayliss and others 1989). The contention that the diffusion of GAG-bearing fragments from the matrix occurs soon after cleavage of core protein is supported by the observation that GAG-rich PG fragments are not found in significant amounts in the matrix (Kimura, Osdoby, Caplan and Hascall 1978).

As intact aggregating PG are not found in significant amounts in the body fluids in contact with the cartilage surfaces, measurement of peptidoglycans in body fluids provides a means of determining the rate of catabolism of cartilage PG. *In vitro* measurement of radioactive ^{35}Sulphate (^{35}S) labelled fragments in incubating ^{35}S-labelled articular cartilage cultures have been used to determine the rate of catabolism or turnover of PG in the matrix (Barone-Varelas, Schnitzer,

Meng and Thonar 1989). Measurement of KS by immunoassay provides a means of measuring cartilage metabolism *in vivo.*

The pathways by which PG fragments are metabolised in the body are unclear. They may be further degraded before entering the circulation, from which they are eliminated via the liver and kidneys (Thonar, Lenz, Klintworth, Caterson, Pachman, Glickman, Katz, Huff and Kuettner 1985). Most KS-bearing molecules in human blood consist of a single KS chain of varying length which is still attached to a small peptide (Thonar and others 1985). Maldonado, Williams, Otten, Flannery, Kuettner and Thonar (1989) showed that KS bearing PG fragments injected intravenously in rabbits are eliminated from the blood at different rates depending on their size and composition (half-life, 6 to 50 minutes). The major pathway of elimination from blood may be by binding of fragments of KS containing terminal galactose residues to galactose receptors on hepatocytes (Schlepper-Schaffer, Hulsmann, Djovkar, Meyer, Herbertz, Kolb and Kolb-Bachofen 1986).

The level of serum KS can vary markedly from individual to individual (Sweet, Coehlo, Schnitlzer, Schnitzer, Lenz, Jakim, Kuettner and Thonar 1988; Seibel, Towbin, Braun, Kiefer, Muller and Paullson 1989). Hascall and Glant (1987), suggest that the extent of KS sulphation may vary considerably from person to person and that this could partly contribute to the wide range of values (53 to 1,009 ng/ml) observed in human patients (Thonar, Manicourt, Williams, Lenz, Sweet, Schnitzer, Otten, Glant and Kuettner 1991a). Levels of KS vary predictably with age (Thonar, Pachman, Lenz, Hayford, Lynch and Kuettner 1988b), rising progressively in the first four years of life in humans, dropping markedly at twelve years of age and continuing to decrease towards adult concentrations. The serum level of cartilage core protein epitope has a similar variation and in children shows a strong correlation with the level of serum KS. This adds strength to the opinion that the level of serum KS can be used to obtain a good measure of the rate of PG catabolism. PG and KS content remain relatively constant with increasing age in adult humans (Thonar, Williams, Maldonado, Lenz, Sweet, Schnitzer, Campion and Kuettner 1991b) and it is concluded therefore that measurement of the level of serum KS can be used as a measure of basal PG turnover in individuals without cartilage disease *in vivo.*

2.2 Turnover of articular cartilage in health and disease.

Maintenance of both the quality and quantity of articular cartilage matrix components and adaptation to functional demands is the result of a balance between the synthetic and catabolic activity of the chondrocyte. The chondrocyte is actively involved in the metabolism of PGs throughout the lifetime of the tissue (Handley, Mc Quillan, Campbell and Bolis 1986).

PGs in cartilage are constantly being turned over. Cartilage PGs turn over more rapidly than the collagen matrix in which they are embedded. Collagen turnover times are very slow; one hundred and twenty years in canine articular cartilage and three hundred and fifty years in human articular cartilage (Akizuki, Mow,

Muller, Pita, Howell and Manicourt (1986). There are fast and slow pools of PG turnover; the overall turnover time in adult canine and lapine articular cartilage is approximately three hundred days and in the human hip joint it is eighteen hundred days (Todhunter and Lust 1992).

The degradation of PGs during normal turnover is regulated by the chondrocytes, which release proteolytic enzymes into the extracellular milieu. PGs and hyaluronan have similar half-lives, suggesting that the PGs within an aggregate are usually turned over as a unit. To maintain the balance, any normal or increased loss of proteoglycan from the matrix must be compensated for by *de novo* synthesis of PG by the cells within the tissue. If this compensatory synthesis fails, then the resistance of the tissue to load will decrease. It has been shown experimentally that healthy chondrocytes monitor the matrix content of PG and can respond rapidly to changes in their concentration in the extracellular matrix (Thomas 1956). This is believed to be mediated by cell surface interactions (Handley and Lowther 1977). There are many other endogenous and exogenous factors that may alter the rate of synthesis of matrix components by chondrocytes (Treadwell and Mankin 1979).

The turnover of normal articular cartilage is effected through a degradative cascade, for which many authors think the driving force is interleukin-1 (IL-1). This cytokine can stimulate production of, and secretion into the matrix of latent collagenase, latent stromelysin and latent gelatinase. It has been demonstrated *in vitro*, that after addition of IL-1 to cartilage explants, release of PGs into the culture medium occurs within hours (Saklatvala, Pilsworth, Sarsfield, Gavrilovic and Heath 1984). It can also stimulate production of prostaglandin E_2, a mediator of acute inflammation.

IL-1 is produced by the mononuclear cell system, including synovial lining cells in the joint, and is also synthesised by chondrocytes as an autocrine activity. IL-1 activity may be controlled both by alterations in its production and alterations to endogenous levels of inhibition of its activity (Wood, Ihrie, Dinarello and Cohen 1983). Articular cartilage also contains a family of neutral metalloproteinases capable of degrading PG (Brandt 1991). Net expression of proteolytic activity of neutral metalloproteinases represents a balance between activation of their latent forms and inhibition of the activity by inhibitors (Figure 3).

The balance of the system lies with at least 2 inhibitors, tissue inhibitor of metalloproteinase (TIMP) and plasminogen activator inhibitor (PAI-1), both of which are synthesised by the chondrocyte and limit the degradative activity of active neutral metalloproteinases and plasminogen activator respectively (Brandt 1991). Conversion of latent stromelysin to active, highly destructive proteases by plasmin provides a second mechanism for matrix degradation. Cathepsin B and D (Bayliss and Ali 1978) are also very effective at degrading cartilage PG as are oxygen free radicals synthesised by the chondrocytes.

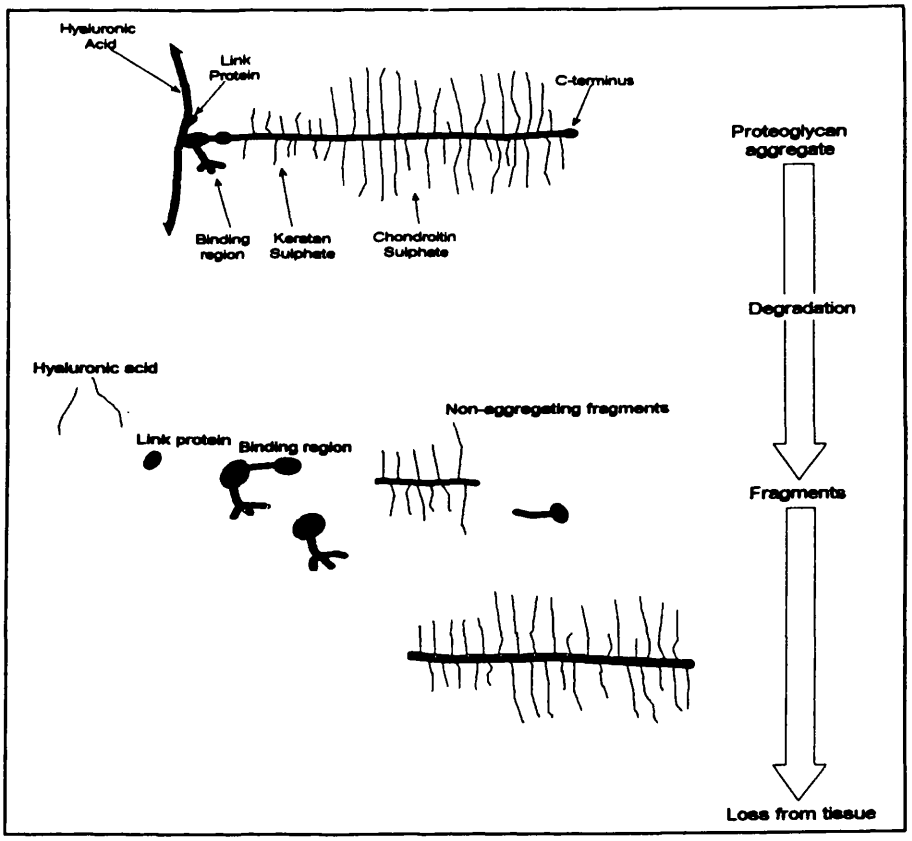

Figure 2

Proposed scheme for the metabolism of proteoglycan and release from the joint

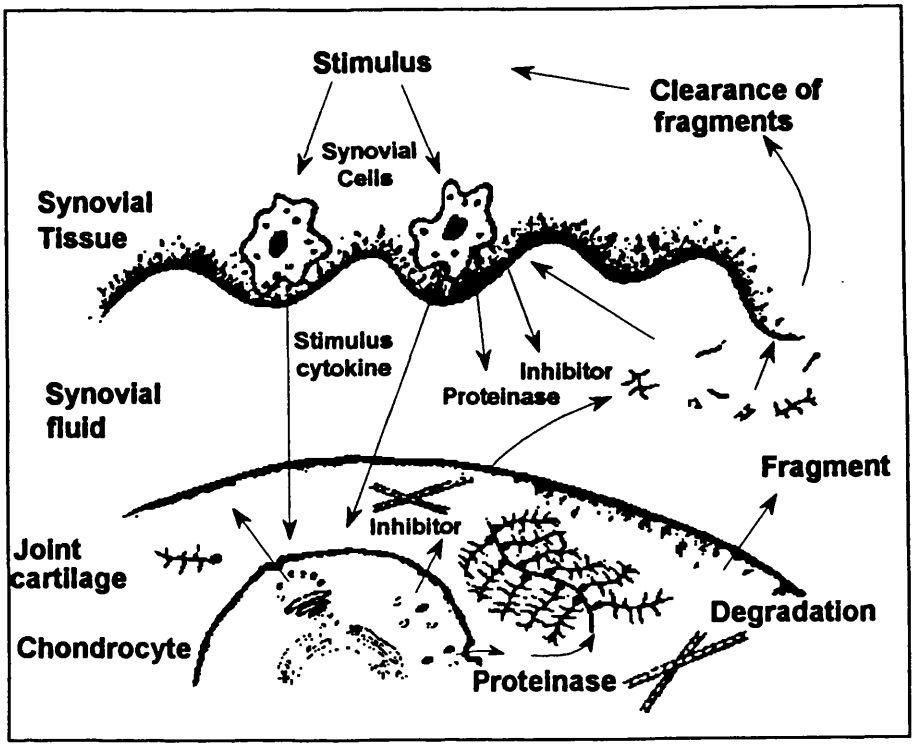

Figure 3

Turnover of proteoglycans: role of proteinases in proteoglycan degradation

2.3. Repair of articular cartilage

An increasing number of growth factors found to influence the metabolism of chondrocytes have been identified (Handley, Lowther and Mc Quillan 1985). Insulin-like growth factor (IGF-1) appears to be one of the strongest factors for a physiological role in the regulation of cartilage metabolism by being present in normal serum and causing a marked stimulation of PG synthesis (Burch, Weir and Van Vyk 1988). Transforming growth factor beta, (TGF ß), has a paracrine and autocrine regulatory function and may play a role in matrix repair in OA (Brandt 1991). It is currently unknown whether age-related changes in the cartilage influence IGF-1 activity, or affect the responsiveness of the chondrocyte to these mediators.

In addition to its responsiveness to cytokines and a variety of other biological mediators, it is clear that chondrocyte metabolism can also be modulated directly by mechanical loading. Support for the ability of cartilage chondrocytes to respond directly to altered mechanical loading of the joints is given by animal models. Increased loading of the joints leads to increases in PG biosynthesis (Caterson and Lowther 1978). Prolonged immobilisation of joints may lead to degenerative changes and arthrosis (Eronen, Videman, Friman and Michelsson 1978). Whereas static loading and prolonged cyclic loading inhibit synthesis of PG and protein, loads of relatively brief duration may stimulate matrix biosynthesis (Gray, Pizzanelli, Grodinsky and Lee 1988).

Little information is available about the *in vivo* rate of release of the PG fragments from joint cartilage. Experiments with cartilage explants *in vitro* indicate that the majority of the PG fragments generated by proteolytic breakdown are released from the tissues with only a small proportion (10 per cent or less) being taken up by the cells of the tissue (Hascall, Morales, Hascall, Handley and Mc Quillan 1983).

3. OSTEOARTHRITIS

3.1. The pathogenesis of osteoarthritis

Osteoarthritis is a slowly progressing multifactorial disease of obscure aetiology. It has been argued that the OA process is a fundamental aspect of joint function. This function is as of yet not fully understood, but a widely held opinion is that it is part of the reparative response to joint injury (Jones and Doherty 1992).

Studies in humans have shown that there is an increased frequency of OA with age (Lane and others 1993). It has therefore been suggested that OA is a manifestation of aging in the joint, possibly reflecting senescence of joint function or the response to cumulative injury (Brandt 1981). However, it has been demonstrated that there are biochemical differences between old and osteoarthritic cartilage, implying that the explanation of aging may be too simplistic (Brandt 1988). In horses, animals as young as two years old may show

evidence of advanced OA in several joints, leading to the suggestion that these animals may have an inherited biochemical abnormality of cartilage (May 1993 unpublished). Todhunter and Lust (1992) comment on the fact that horses presenting with joint disease range from two years old to aged, suggesting that aging is not the primary cause of OA. Although certain studies in humans refute that mechanical factors correlate with OA (Panush, Smith, Caldwell, Edwards, Longley, Yonker, Webster, Nauman, Stork and Petterson 1986; Lane, Block, Jones, Marshall, Wood and Fries 1986), repetitive trauma has been shown to be an epidemiological factor in the development of the disease in humans (Peyron 1989).

One of the earliest biochemical changes occurring in osteoarthritic cartilage appears to be related to structural alterations in PG and collagen and the total content of these macromolecules in the cartilage. Although wear may be a factor in the loss of cartilage, strong evidence supports the concept of lysosomal and neutral metalloproteinases, (e.g. stromelysin, collagenase, gelatinase) accounting for the majority of the loss of cartilage matrix in OA. Increased levels of metalloproteinase have been found in osteoarthritic cartilage compared to controls (Martel-Pelletier, Pelletier, Cloutier, Howell, Ghandur-Mnaymneh and Woessner 1984). Cartilage fragments and other particles have the ability to stimulate production of neutral metalloproteinase and prostaglandin E_2 by chondrocytes and synovial cells *in vitro* (Cheung, Halverson and Mc Carthy 1981). This forms a direct route by which the synthesis of enzymes responsible for cartilage degradation may be stimulated by traumatised joints (Evans, Mears and Cosgrove 1981).

Activation of cells of synovial membrane by e.g. trauma, may lead to production of IL-1 by activated cells which would stimulate the production of prostaglandin E_2 and neutral metalloproteinases by chondrocytes, leading to pain and cartilage erosion. Increased production of cytokines in disease is due to lymphocytic activation, but can also be triggered by microbial products such as bacterial lipopolysaccharide (Arend and Massoni 1986). IL-1 and tumour necrosis factor (TNF) interact synergistically, and may induce their own and each other's synthesis (Krane 1990) in addition to that of other cytokines, thereby providing a mechanism by which inflammation may persist in a joint. Endogenous inhibitors of IL-1 have been detected in serum, urine and cell culture supernatants in humans and appear to function as physiological modulators of IL-1 (Rosenstreich, Yost, Tu and Brown 1987). It has been demonstrated that synovial fluid from horses with acute OA has inhibitory effects on IL-1 and IL-ß stimulation of prostaglandin E_2 production by equine synovial cells *in vitro* (May, Hooke and Lees 1992). Types or levels of inhibitors may vary in time with the course of the disease.

Biochemically, early structural alterations in osteoarthritic cartilage include a reduction of PGs, increased water content, chondrocyte cloning and an increased synthesis of matrix (Mankin, Johnson and Lippielo 1981). The new cells are very active metabolically, producing increasing quantities of collagen, PG and

hyaluronan, although these new products have a reduced ability to form aggregates in general (Martel-Pelletier and others 1984). Prior to the loss of cartilage mass and PG depletion, marked biosynthetic activity (repair) of the chondrocytes may be associated with thickening of the cartilage in the earlier stages of OA. In the canine cruciate deficient model of OA, autoradiography with radiolabelled sulphate has shown that all cell have enhanced biosynthetic activity. (Thonar and others 1991a). This has been termed the hypertrophic repair phase of OA (Brandt 1991).

The degradation of articular cartilage is initially focal but may involve diffuse areas of the cartilage surface in advanced cases. As the disease progresses, the cartilage becomes ulcerated and there is development of fibrillation in the superficial layer, together with fissuring, osteophyte formation and sclerosis of the subchondral bone. Anabolic processes no longer keep pace with catabolism of the cartilage, resulting in an osteoarthritic joint.

3.2 Classification of osteoarthritis

Much confusion exists in both human and veterinary literature as to the classification of OA/degenerative joint disease. In veterinary literature, Mc Ilwraith (1987) and May (1993, unpublished) have devised classification systems for the condition in horses (Table 1). Poole and Meagher (1990) describe OA in the horse as being primary, i.e. an idiopathic, age-related disease of insidious onset in which there is chronic deterioration of normal joint structure and loss of function, or secondary, i.e. occurring in previously normal joints following injury of these joints. In secondary OA, injury of the joint may occur at any stage following the primary insult. Another method of classifying OA is based on the possible adverse effects of biomechanical forces on normal and abnormal articulations (Mitchell and Creuss 1977). This scheme only recognises secondary causes of DJD. Irrespective of the classification system used, it is clear that degeneration and loss of the articular cartilage with concommitant joint failure as described above is the common end-point of this condition.

Degenerative joint disease entities

Type

1. Young, unbroken (2 years old) horses, advanced DJD in several joints. Possible inherited biochemical abnormality of cartilage.
2. Young racehorses, 2 to 4 years old. Acute disease in highly mobile joints.
3. Horses in light work, 4 to 8 years old. Insidious disease, distal limb joints with and without navicular disease.
4. Older horses, insidious disease of low motion joints.
5. Older horses, age changes unrelated to lameness.

May S.A. (1993)
(unpublished)

Degenerative joint disease entities

Type

1. Acute, associated with synovitis and high motion joints.
2. Insidious, associated with low motion joints.
3. Incidental or non-progressive articular cartilage erosion.
4. Secondary to other identified problems.

a. Intra-articular fractures.
b. Dislocations / ligamentous rupture.
c. Wounds.
d. Septic arthritis.
e. Osteochondrosis.

5. Chondromalacia.

Mc Ilwraith C.W. (1987)

Table 1
Classification of degenerative joint disease entities in the horse.

3.3 Diagnosis of osteoarthritis

Apart from clinical signs such as pain, stiffness, and joint distension, radiography is the mainstay in the diagnosis of OA. In human medicine, the Kellgren and Lawrence grading system (1957) has been used in research on OA for over thirty years. This system relies heavily on radiographic evidence of osteophytes, with subsequent increase in grade being dependant on the concomittant loss of joint space radiographically. However, osteophytes may be a natural function of aging, in that articular cartilage alters its biomechanical makeup with age and the altered environment may favour degeneration of the articular cartilage and osteophyte formation (Hammerman 1989). Altman, Fries, Bloch, Carstens, Cooke, Genant, Gafton, Groth, Mc Shane and Murphy (1987) found that joint space narrowing, followed by assessment of osteophytes were the best variables in judging the sequence of events. The correlation between symptom and structure is, however, poorly understood. Loss of 30 to 50 per cent of bone substance is required before radiographic detection of osteolysis is possible (Lodwick 1964). Thus, degenerative changes are well advanced before being detected radiographically. It has been shown that up to 40 per cent of humans with radiographic evidence of OA are asymptomatic (Lane and others 1993). In horses, Jeffcott (1983) detected small bone spurs in a number of joints in a significant percentage of animals in regular work with no evidence of lameness. The lack of a clear relationship between clinical signs and presence or absence of radiographic abnormalities continues to be a problem for clinicians.

Christiansen (1985), using bone scintigraphy in human studies showed that only active non-specific joint involvement could be demonstrated and that this did not necessarily reflect progressive degenerative joint disease. Diagnostic arthroscopy and contrast arthrography are techniques also used in the diagnosis of equine joint disease. However, these techniques are invasive and animals are presented usually at a time when pathology within the articular cartilage is already well advanced. Arthroscopic and pathological studies serve to highlight potentially very important discrepancies between plain radiographic images and the actual situation within the joint in both humans (Fife, Brandt, Braunstein, Katz and Shelbourne 1991) and horses (Nillson and Olsson 1973).

4. MARKERS OF JOINT DISEASE

4.1 Biochemical Markers of Joint Disease

From a diagnostic and therapeutic viewpoint, it would be preferable to have convenient and economical non-invasive tests that would indicate the metabolic or functional state of the synovium and cartilage at an early stage in the disease, before the onset of radiographic changes. There are several different sources and types of markers that have potential for use in the diagnosis of joint disease. These potential markers can be (a) anabolic markers, i.e. molecules that are only newly synthesised by the chondrocyte during the process of repair and remodelling (b) catabolic markers, i.e. those that are breakdown products or activated enzymes that cause release of these degradation products and (c)

general markers that occur as a consequence of increased catabolic and anabolic processes in the disease.

A number of assays for biochemical markers have been evaluated in the investigation of articular cartilage metabolism. Assays used for detection of joint disease focus on both synovial membrane and cartilage metabolism. Hyaluronan measurement has been carried out in humans (Goldberg, Lenz and Thonar 1988; Hedin, Weitoft, Hedin, Engstrom-Laurent and Saxne 1991) and horses (Hilbert, Rowley and Antonas 1984). Hyaluronan, however, is not specific to cartilage or to joints. Fibronectin has been measured in equine joint disease (Todhunter, Lust, Freeman and Parente 1990), and reported to be a useful indicator of joint disease severity and activity. IL-1 activity has been measured in equine joints as an indicator of disease activity (May and others 1992). Synovial fluid lysozyme activity correlates well with experimental inflammation or joint injury in horses (Torbeck and Prieur 1979). Collagenase activity (measured using synovial aldehyde groups) correlates with equine articular cartilage lesions at macroscopic level (Maldonado, Garces, Auba and Horvath 1983). Collagen breakdown products have been studied in the horse as potential markers of joint disease (Price, Colwell, Eastell, Goodship and Russell 1992). Antibodies against Type two collagen have been detected in synovial fluid and serum from humans (Mollenhauer, von der Mark, Burmester, Gluckelt, Lutjen-Drecoll and Brune 1988) and horses with OA (Niebauer, Wolf, Yarmush and Richardson 1988).

4.2 Use of keratan sulphate as a biochemical marker in the evaluation of articular cartilage health status

The rational for measuring KS is based on the fact that more than 95 per cent of the mass of KS in the body is located in hyaline cartilage, and as such, it is reasonably cartilage specific (Thonar, Williams, Sweet, Maldonado, Lenz, Schnitzer and Kuettner 1989a). A polyclonal antiserum reacting with KS was initially characterised by Conrad, Ager-Johnson and Woo (1982) and shortly thereafter, mouse monoclonal antibodies against KS were isolated (Funderburgh, Stenzel-Johnson and Chandler 1983; Caterson, Baker and Christner 1983). Several other monoclonal antibodies against KS have subsequently been isolated, and have been shown to be highly specific for KS epitopes (Caterson and others 1983; Ratcliffe, Doherty, Maini and Hardingham 1988). These antibodies have been used in the immunohistochemical localisation of KS in embryonic tissues (Hyldahl, Aspinall and Watt 1986; Funderburgh, Caterson and Conrad 1986) and via immunoassay for the detection of KS in human serum (Thonar and others 1985) and equine serum and synovial fluid (Alwan, Carter, Bennet, May and Edwards 1990; Todhunter, Yeager, Freeman, Parente and Lust 1993).

The enzyme linked immunosorbent assay (ELISA) used in the current study is based on that of Thonar and others (1985), with several minor modifications (Thonar and others 1991b). The assay uses a mouse monoclonal antibody (MAb) (1/20/5/D4) (Caterson and others 1983). This antibody specifically recognises a sulphated antigenic determinant in the polysaccharide structure of both corneal

and skeletal KS. It was shown by Caterson and others (1983), that the MAb did not recognise antigenic determinants on PGs isolated from Swarm rat chondrosarcoma. This finding is consistent with several biochemical analyses showing the absence of KS in PGs synthesised by this tissue. The MAb did not recognise determinants present in other GAGs such as dermatan sulphate, heparin sulphate, heparin or hyaluronan.

4.3. Studies on KS as a marker of cartilage catabolism in joint disease

Quantitative and qualitative analyses of fragments of cartilage PG in blood and synovial fluid have provided important information on the catabolism of articular cartilage PG in a single synovial joint. The contention that most of the KS molecules in blood represent degradation products of cartilage PGs is supported by studies such as that of Block, Schnitzer, Andersson, Lenz, Jefferey, Mc Neill and Thonar (1988), wherein papain-induced chemonucleolysis of an intervertebral disc resulted in a significant increase in the concentration of KS in serum, compared to patients who had undergone laminectomy. Similarly, in the study of Williams, Downey and Thonar (1988), papain-induced destruction of articular cartilage in a single joint in rabbits was accompanied by a 3 to 5 fold increase in the concentration of KS measured in serum, lasting 48 hours, before progressively decreasing to baseline values at nine days post-injection.

Levels of cartilage PG epitopes measured in synovial fluid reflect the metabolism/catabolism of PG within that joint. It can be argued that measurement of PG epitopes in blood represents the average of what is occurring in all cartilages in the body. However, the studies of Block and others (1988) and Williams and others (1988) discussed above suggest that extensive degradation of PG within a single joint can be detected by changes in serum PG concentrations.

Witter, Roughley, Webber, Roberts, Keystone and Poole (1987) demonstrated that synovial fluids collected from arthritic joints contained significant amounts of PG fragments of different sizes and composition. It was found that the synovial fluid from patients with reactive arthritis had much higher levels of PG epitope than that from patients with rheumatoid arthritis, leading Saxne, Heinegard and Wollheim (1986) to suggest that measurement of the levels of epitope in synovial fluid could be used diagnostically. These authors also showed that the levels of PG epitope decreased significantly over time following intra-articular injection of glucocorticoid. This lead to the suggestion that measurement of the level of PG epitope over time could be used to monitor the progress of the disease. Lohmander, Dahlberg, Ryd and Heinegard (1989), using the same assay also demonstrated that joint trauma caused the release of cartilage PG fragments into the synovial fluid, and suggested that in the acute phase after injury, synovial fluid levels of PG epitope could be related to the severity of the disease, but in the later stages, they may reflect the degree of chronic joint instability .

Studies by Lohmander and others (1989), showed that high synovial fluid concentrations of PGs were found in acute inflammatory conditions e.g. reactive arthritis, but that there was a broad range of lower concentrations in chronic

conditions. However, as the chronicity of the latter conditions was not staged, there may have been a number of different subgroups within the range. Ratcliffe and others (1988) have shown that in the acute stages of joint disease, there is a rapid increase in PG concentration in synovial fluid, however, this is not sustained during non-active stages. In more chronic joint diseases, the concentrations of PGs in synovial fluid were lower than in acute disease and showed little apparent correlation with clinically assessed disease activity. The failure to detect greatly increased concentrations in most patients with chronic disease may be due to several factors, e.g. the activity involved in the rate of release of PG may be less than in acute disease. If there are episodes of rapid release in chronic disease, these may be of short duration or infrequent, or both, or may precede clinical signs, such that they do not coincide with clinical sampling. Finally, if only small changes occur, these may be masked by the broad range of natural variation in PG concentration that is evident in the patient population.

4.4. Use of KS as a marker of generalised cartilage catabolism

Thonar and others (1985) demonstrated that patients with OA had significantly higher circulating levels of KS than patients hospitalised for conditions other than joint disease (control population). It has also been elucidated that a significant proportion of patients with OA do not have elevated levels of the KS epitope while a small percentage of adults without any clinical signs of OA do have elevated KS levels (Thonar and others 1991b).

Results of several studies, (Thonar, Schnitzer and Kuettner 1987; Sweet and others 1988) have shown that a single measurement of the concentration of serum KS is not very useful in the assessment of cartilage destruction in arthritic joints. The large range of serum KS in human patients hinders the usefulness of the KS assay as a diagnostic test for OA or its subsets (Thonar and others 1988a).

It is postulated that apparently normal individuals with significantly higher than average KS levels may be at risk of developing OA subsequently and that the persistent elevation of KS in these individuals for a prolonged period following joint injury may be indicative of a generalised disturbance in cartilage metabolism. This hypothesis has been compounded by the result of several studies (Sweet and others 1988; Brandt and Thonar 1989). In the study of Sweet and others (1988), patients with hypertrophic OA were shown to have significantly higher serum KS levels than adults without joint disease. These patients generally had multiple joint involvement. It was also noted that they had significantly elevated levels of KS six months following the removal of a degenerate joint (hip joint). This suggested that abnormal PG degradation in the degenerate hip joint was not a major influence on the high preoperative KS level and that a more generalised disorder of cartilage was present.

Additional support for the concept of a systemic or generalised increase in the rate of metabolism of cartilage PG in OA is found in the studies of Brandt and Thonar (1989), and Manicourt, Lenz and Thonar (1991). In experimentally

induced canine OA, transection of the anterior cruciate ligament caused a rapid increase in serum concentration of KS which remained elevated until twelve weeks post surgery (Manicourt and others 1991). This study showed that the level of KS epitope had risen by day seven and remained elevated for at least thirteen weeks post-operatively. The level of KS epitope in the synovial fluid of the operated joint was several times higher than in serum and correlated with serum levels of the epitope. Sham operation was not accompanied by any change in the level of serum KS at any time after surgery. This finding suggested that a significant proportion of the KS bearing fragments appearing in the serum after transection of the anterior cruciate ligament are derived from an increase in the rate of catabolism of articular cartilage PG within the operated joint. However, the relationship of this elevation to the subsequent development of OA has not been fully elucidated.

In the study of Brandt and Thonar (1989) similar results were obtained using the same model of joint disease. The PG content of the operated knee did not change during this time, suggesting that the increase in metabolic activitiy of the chondrocytes was restricted to the cartilage in the operated knee. However, the magnitude of the rise in serum KS suggested that it was more systemic or generalised in nature.

Measurement of the levels of serum KS epitope in models of OA such as the anterior cruciate ligament transection model have been suggested as a useful non-invasive tool for the study of the effects of chondroprotective agents and other drug treatments in the prevention of post-operative OA changes in this model (Pelletier and Martel-Pelletier 1989).

4.5 Studies on keratan sulphate as a marker of joint disease in the horse

There is a relative paucity of information on KS in the horse compared to human literature. Efforts in veterinary medicine have primarily been concentrated on the use of KS as a marker for OA and other forms of joint disease in the horse.

Previous investigators have studied GAGs in synovial fluid, serum and urine of both normal horses and those with various forms of joint disease. Little, Hilbert, Wickstrom and Hedlund (1990), found no difference in GAG levels in synovial fluid from horses with OA and normal horses, whereas Alwan, Carter, Bennet and Edwards (1990) reported high levels of GAGs in synovial fluid, serum and urine of horses with OA compared with normal horses. Levels of GAGs in horses with septic arthritis were lower than those reported in OA (Alwan and others 1990). In their study, high levels of GAGs were also found in infectious and traumatic arthritis and OA and therefore, could not be considered as a specific marker for OA. The limitations of measuring GAGs as indicators of articular cartilage pathology are highlighted also by the fact that they are synthesed by synovial tissue (Hammerman, Smith, Keiser and Craig 1982; Marsh, Wiebkin, Gale, Muir and Maini 1979) and therefore are neither restricted to PGs or specific for cartilage compared to KS.

In another study, Alwan, Carter, Bennet, May and Edwards (1990), investigated articular cartilage breakdown using an ELISA for KS. They also measured sulphated GAGs (S-GAGs) using the dimethylene blue (DMB) assay of Farndale, Buttle and Barret (1986). The latter assay has the disadvantage of measuring all the released S-GAGs whether they are from cartilage or other tissues in the joint. This study demonstrated that a significant proportion of horses with OA had elevated levels of KS both in articular cartilage and serum compared to their group of normal horses. Serum levels of KS were found to be much lower than synovial fluid levels and there was no correlation with joint measurements. There was no correlation between the presence of neutrophils in synovial fluid and KS levels. A good correlation was found between synovial fluid measurements of S-GAGs and KS. However, as stated above, this does not imply that they are measuring identical pathological mechanisms. They concluded that synovial fluid KS measurements had potential for use as markers of degradation of cartilage in OA.

In a study by Yovich, Carroll and Bell (1991), KS concentrations in synovial fluid from the midcarpal joint of horses were measured using the modified (Carroll, Stuart, Armstrong, Breidahl and Laing 1991) DMB assay of Farndale and others (1986). The horses presented for lameness referrable to this joint were lame on trotting and had synovial effusion. Intra-articular anaesthesia of the affected joint relieved the lameness. Results of this study showed that in the group of lame horses, there was no difference in synovial fluid KS levels compared to normal horses.

Todhunter, Yeager, Freeman, Parente and Lust (1993) used KS as a marker of hyaline cartilage catabolism in the evaluation of the response of cartilage to surgically created focal osteochondral defects or generalised chemical destruction using chymopapain. They also evaluated the effects of post-operative exercise and intra-articular medication with polysulphated glycosaminoglycan (PSGAG) in this controlled study. Their findings indicated that there was a greater increase over baseline in KS measurements from synovial fluid and serum in chymopapain-injected joints (generalised cartilage destruction) compared to joints with focal osteochondral defects. In their study, the concentration of KS in synovial fluid was not related to the development of OA in ponies. Exercise or medication did not have an effect on plasma KS concentration and there was no correlation between synovial fluid and plasma KS concentration in any of the animal groups. The results of these experiments showed that KS concentration may be increased in acute, generalised marked articular cartilage catabolism and that the turnover of KS in cartilage with large osteochondral defects was affected by intra-articular medication with PSGAG. KS did not seem to be a definite marker of localised catabolism of aggregan as occurs in the later stages of OA. They concluded that changes in KS concentration may reflect generalised rather than focal hyaline cartilage catabolism.

To date, studies in horses have concentrated primarily on the use of KS as a marker in the diagnosis of OA (Alwan and others 1990; Yovich and others 1991). The study by Todhunter and others (1993) is the first to critically evaluate KS as a marker of the effect of treatment and exercise in a model of equine joint disease. It has been shown in humans by Block and others (1989) that the level of serum KS does not fluctuate diurnally or from day to day, however it has not been ascertained whether this is the case in the horse. In humans, it has been noted that moderate (Block and others 1989), or even strenuous exercise, as in running a marathon (Thonar and others 1991) did not result in significant changes in serum KS levels. Studies on synovial fluid GAGs in humans with joint disease have not included subjects in a daily vigorous exercise routine. The effect of exercise in racing/riding horses on the turnover or clearance of KS from synovial fluid or serum has not been examined. In studies carried out in both humans and animals, evaluating KS levels in joint disease, the contribution of age-related breakdown of articular cartilage in habitually unloaded regions that occurs in virtually every diarthrodal joint has not been taken into consideration (Brandt 1991). It is not known to what extent this may contribute to the serum concentration of a marker in normal and osteoarthritic subjects.

5. AIMS OF THE STUDY

The objectives of this project were as follows:

(1) To establish and validate an enzyme-linked immunosorbent assay (ELISA) for use in our laboratory to evaluate KS levels in equine synovial fluid and serum.

(2) To quantify KS levels in synovial fluid from carpal and tarsal joints and serum in a group of "normal" horses.

(3) To investigate whether there is (a) diurnal and (b) day to day variation in serum KS levels in normal ponies.

(4) To assess if serum KS levels in a group of racehorses in training are affected by a period of acute exercise.

(5) To monitor synovial fluid KS levels in three cases of degenerative joint disease and to evaluate longitudinal measurements of serum KS in these animals post-operatively / in the recovery phase.

CHAPTER II
GENERAL METHODS

1. PURIFICATION OF PROTEOGLYCAN A1D1 MONOMER BY DENSITY GRADIENT CENTRIFUGATION

Cartilage PG may be extracted either by a disruptive procedure with high speed homogenisation of the tissue or a dissociative procedure, involving extraction with a high-ionic strength salt solution (Hascall and Sadjera 1969).
The latter technique is favourable as it minimises enzymatic and mechanical fragmentation of the macromolecules (Dunstone and Cleland 1975). This method of extraction releases 80 per cent of the tissue PGs, which contain 87 per cent CS, 6 per cent KS and 7 per cent protein (Hascall and Sadjera 1969). Subsequent fractionation of the extract using density gradient centrifugation allows the isolation of approximately 95 per cent of the extracted PG as PG subunit.

1.1 Principles of associative and dissociative density gradient centrifugation

Extraction of PG with 4 M guanidine hydrochloride (GHCl) results in a solution that contains both aggregated and non-aggregated molecules (Hardingham and Muir 1974). In the study of Hardingham and Muir (1974), dissociation of purified PG aggregates was shown to release an interacting component of buoyant density higher than that of the glycoprotein-link fraction of Hascall and Sadjera (1969). Equilibrium density gradient centrifugation of the purified PG from bovine nasal septum cartilage in the presence of 4 M GHCl was shown to dissociate aggregates and separate a protein-rich fraction from the majority of the PG (Hascall and Sadjera 1969). This protein-rich fraction contained hyaluronan. Most of the hyaluronan in cartilage is associated with PG aggregates (Hardingham and Muir 1974).

Purification of PG monomer from bovine nasal cartilage is carried out in a two stage process. First, tissue slices are extracted with 4 M GHCl. The caesium chloride is added directly to the clarified extract and an equilibrium density gradient is established in an associative gradient. The associative step is carried out first to purify PG and hyaluronan.

Fractionation in an associative gradient results in a fraction, A1 (Figure 4), that contains PG and hyaluronan, and a fraction, A2, that contains collagen and is protein-rich. This is followed with a dissociative procedure to obtain PG monomer. Fractionation in a dissociative gradient separates non-covalently bound protein from purified PG. The fraction D1 will contain hyaluronan and non-covalently bound protein and the fraction D2 will contain disaggregated PG (Figure 4). All procedures are carried out in the presence of protease inhibitors to ensure that intact PG monomer is obtained.

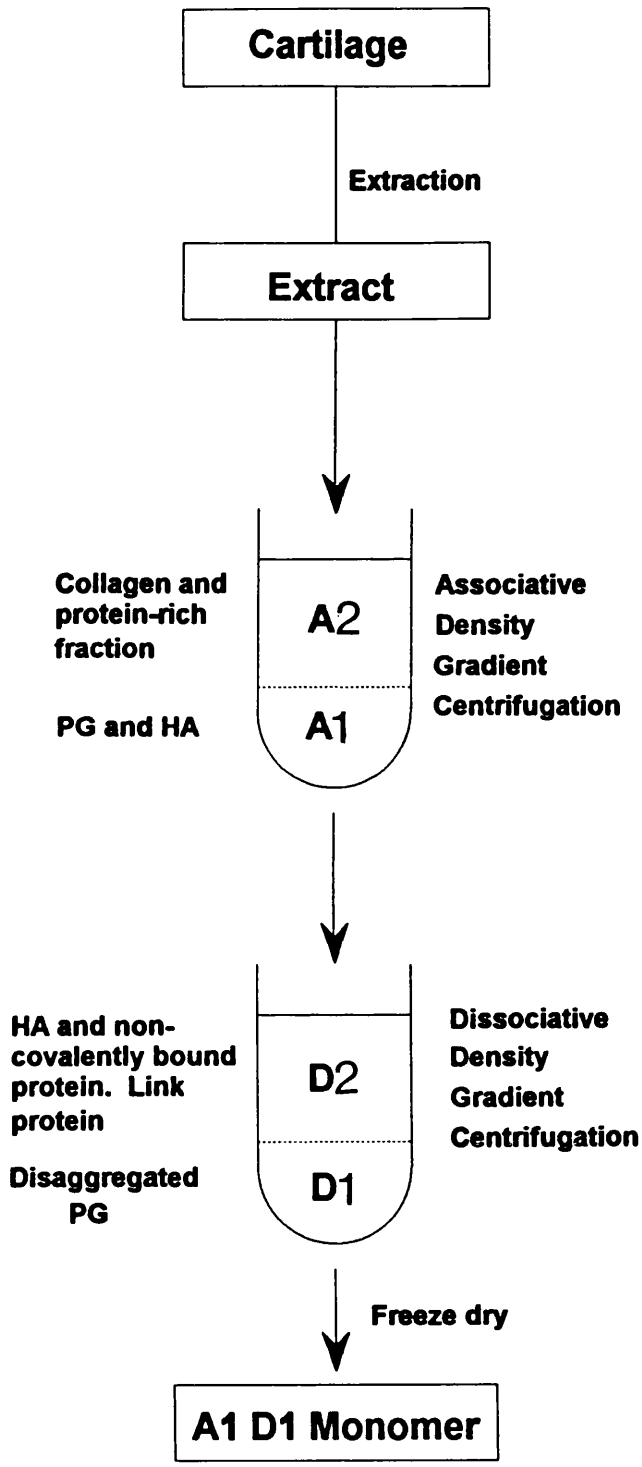

Figure 4

Principle of associative and dissociative density gradient centrifugation.

1.2. Materials and methods

All chemicals used were analytical grade. Suppliers of reagents are listed in Appendix 1. Details of the composition of buffers used at the various stages are contained in Appendix 2.

Bovine nasal septum cartilage (6.6 g wet weight) was obtained from a two year old animal within 30 mins of death and placed in a Tris buffer (Trizma base, 0.05M, sodium chloride 0.15M, sodium azide (w/v) 0.01 per cent, ethylene diamine tetraacetic acid (EDTA) 0.01M, pH 4.3) kept at 4 °C until processing. The cartilage was processed within three hours of collection.

The cartilage was cut into small pieces and homogenised using a Thyristor Regler homogeniser. Proteoglycans were extracted for 65 hours at 4 °C with constant stirring in 100 ml solution of sodium acetate buffer (0.05M sodium acetate trihydrate pH 5.8 (Hascall and Sadjera 1969) with protease inhibitors (EDTA 0.1M, 6-aminocaproic acid 0.1M and benzamidine 0.005M (Oegema, Hascall and Dziewiatkowski 1975) containing 4M GHCl.

Prior to associative density gradient centrifugation (ADGC) the homogenised tissue solution was centrifuged at 2054 *g* for 15 minutes at 4 °C and the supernatant was added to seven times the volume of sodium acetate buffer to make up a final concentration of 0.5 M solution of GHCl.

1.3. Associative density gradient centrifugation

The extract in 0.5M GHCl was adjusted to a starting density of 1.69 g/ml. with solid caesium chloride. 1.19 g of solid caesium chloride per gram of supernate was added (Patel 1981) The theoretical gradient for the rotor was calculated using the method of Ifft, Voet and Vinograd (1961), (Appendix 3) and was between 1.51 g/ml *(p min.)* and 1.85 g/ml *(p max).* The extract was placed in 11 ml polypropylene tubes (Beckman Ltd. U.K) with metal-rubber expander caps. The tubes were centrifuged at 147,500 *g* at 18 °C in a Beckman ultracentrifuge angle rotor (Ti60) for 48 hours. The rotor was allowed to come to rest with the brake off to avoid any vibration disturbing the gradient.

1.4. Fractionation of gradients

Fractionation was carried out using a tube piercer (MSE U.K.). Fractions of approximately 1.1 ml each were collected in 1.5 ml Treff tubes (Scotlabs. U.K.). The densities of the isopycnic gradient profiles created at 48 hrs under ADGC conditions were measured using a density meter (Paar Scientific Ltd.) and are shown in Table 2. Only fractions with a starting density greater than 1.72 g/ml were included (Hascall and Sadjera 1969; Patel 1981). This consisted of the bottom four fractions from each tube. Approximately 95 per cent of the extracted PGs are present at a density greater than 1.72 g/ml, (Hascall and Sadjera 1969). The total volume of solution recovered at this stage was 44 ml.

1.5. Dissociative density gradient centrifugation

Fractions of starting density greater than 1.72 g/ml were pooled and converted to dissociative conditions by adding an equal volume of 7.5M GHCl. Caesium chloride was added empirically to the PG preparation in 4M GHCl to adjust the starting density to 1.52 g/ml (gradient formed during centrifugation, *p min* 1.33, *p max* 1.65) (Appendix 3). The tubes were centrifuged in the same rotors under the same conditions as above. Following ultracentrifugation, the tubes were taken and fractionated as before. The density of each fraction was estimated using a density meter as above, and the bottom four fractions from each tube were pooled (density greater than or equal to1.54 g/ml). The recovery volume was 17.6 ml. Densities of the fractions obtained are shown in Table 3.

The PG solution (8.8ml) was then dialysed (Dialysis membrane, Pierce U.K. Ltd.) initially for 24 hours against sodium acetate buffer and then for 24 hrs against distilled water to remove impurities and salts. The dialysate was lyophylised in plastic graduated Henley tubes (Sarstedt U.K. Ltd.) for 48 hours. The weight of PG monomer recovered was 9.2 mg.

1.6. Results

The density gradients for the associative density gradient centrifugation (ADGC) and dissociative density gradient centrifugation (DDGC) are shown in Figures 5 and 6. They matched well the predicted values calculated from the theoretical gradients. Differences between the actual and theoretical gradients can be explained by the fact that the theoretical gradient is calculated using a pure caesium chloride solution. In the actual density gradient, the densities measured come from a solution which contains GHCl and protein material which contribute to the variation from the calculated gradient.

Fraction	*Tube 1*	*Tube 2*	*Tube 3.*	*Mean.*
10	1.81	1.78	1.80	**1.79**
9	***	1.78	1.79	**1.79**
8	1.74	1.76	1.76	**1.76**
7	1.73	1.74	1.74	**1.74**
6	1.71	1.72	1.72	**1.72**
5	1.69	1.67	1.69	**1.69**
4	1.68	1.67	1.67	**1.67**
3	1.66	1.65	1.65	**1.65**
2	1.64	1.63	1.63	**1.63**
1	1.62	1.62	1.62	**1.62**

***= fraction lost.

Table 2
Densities of fractions obtained from associative density gradient centrifugation.
Individual values of each of the ten fractions from three randomly selected tubes and mean values of each fraction from the three tubes are shown. Densities were measured (g/ml) using a digital density meter (Paar Scientific Ltd.)

Fraction	Tube 1	Tube 2	Tube 3	Tube 4	Mean.
10	1.59	1.59	1.61	1.62	**1.60**
9	1.57	1.58	1.59	1.58	**1.58**
8	1.55	1.57	1.56	1.57	**1.56**
7	1.53	1.54	1.54	1.54	**1.54**
6	1.51				
5	1.48				
4	1.45				
3	1.43				
2	1.41				

Table 3
Densities of fractions obtained from dissociative density gradient centrifugation.
Four tubes were fractionated; of these one tube had every fraction measured. The mean values of the bottom four fractions are also recorded. Densities were measured (g/ml) using a digital density meter (Paar Scientific Ltd.).

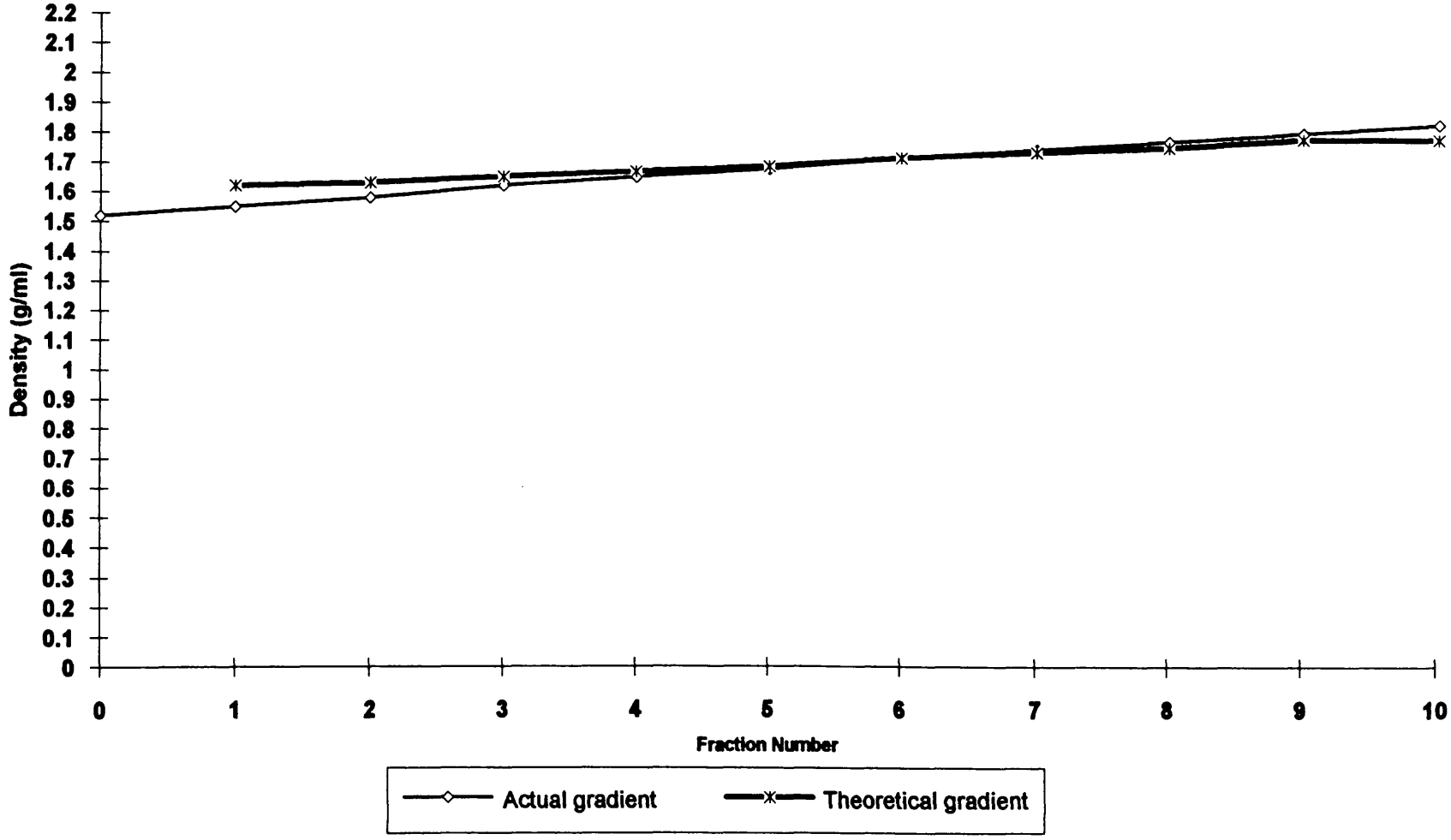

Figure 5
Associative Density Gradient Centrifugation: Actual and Theoretical gradients

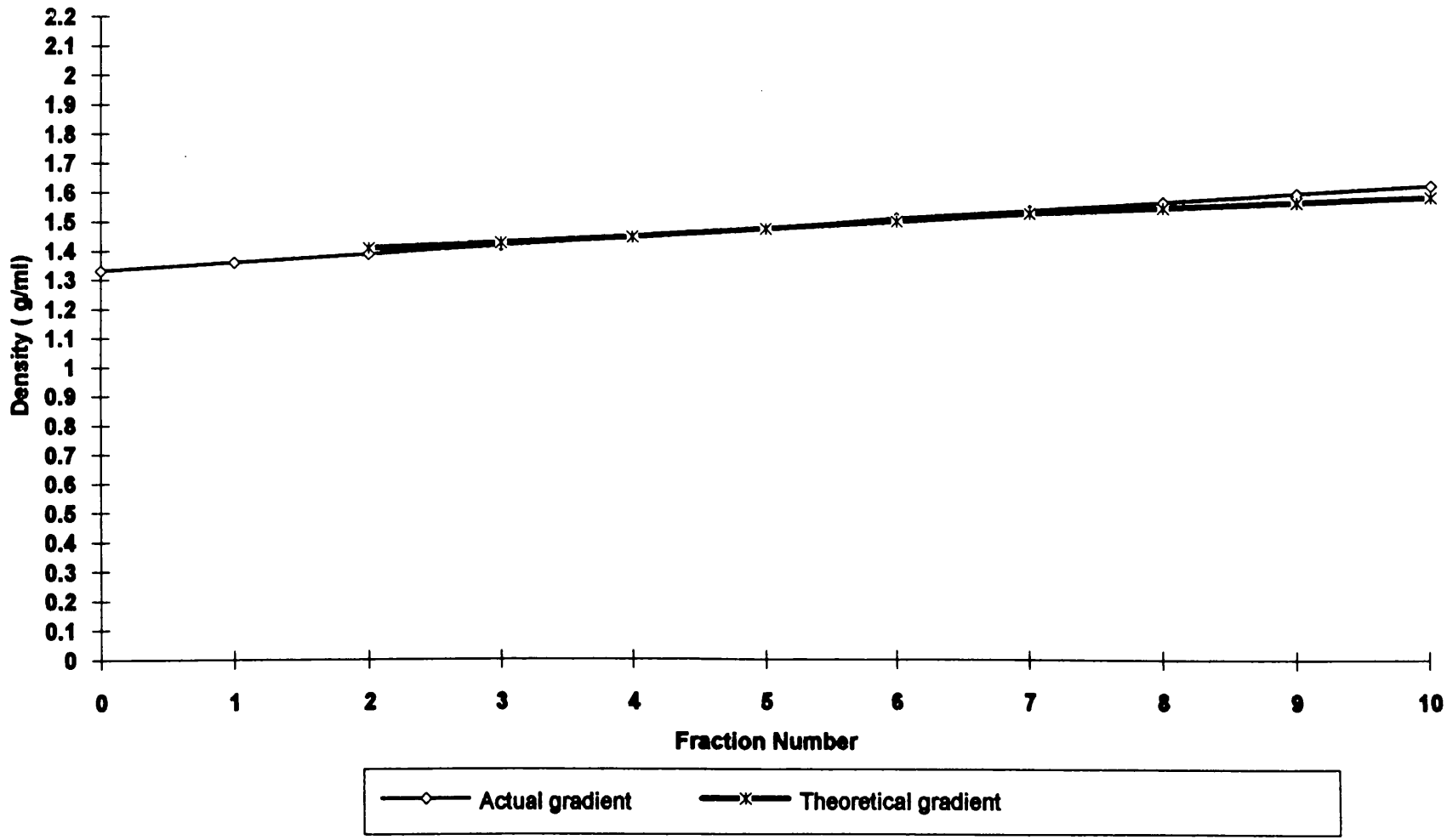

Figure 6
Dissociative Density Gradient Centrifugation: Actual and Theoretical gradients

2. PLATE COATING PROCEDURE

2.1. Materials

Bovine Nasal Septum A1D1 (BNS/ A1D1), the antigen used to coat plates was stored, dessicated at —20 °C. Prior to weighing, the purified PG was thoroughly dried, by lyophylisation for 24 hrs. The same procedure was followed every time BNS/A1D1 was used to ensure uniformity in plate coating.

2.2. Plate coating

Nunc Immuno Plates (A/S Nunc, Denmark) were cleaned by immersing in a 50 per cent ethanol, 50 per cent distilled water solution for 20 mins. The plates were emptied and dried thoroughly.

The chondroitin ABC lyase (ICN Biomedicals Ltd., U.K) was reconstituted by adding 1 ml of chondroitin ABC lyase buffer (0.1M sodium acetate, 0.1M Tris, pH 7.3) (Appendix 4) to 5 units of enzyme. A quantity of 4.8 mg of dried BNS-A1D1 was weighed out, sufficient for 40 plates. The reconstituted chondroitin ABC-lyase was subsequently added to the BNS/A1D1 in the ratio of 0.2 units of chondroitin ABC lyase to 1 mg of BNS/A1D1. The tube was capped and incubated at 37 °C with shaking for 2 hours.

The chondroitin ABC-lyase treated BNS/A1D1 was carefully diluted with prepared coating buffer in the ratio of 1ml of chondroitin ABC lyase to 800 ml of plate coating buffer (20 mM sodium carbonate, 20 mM sodium bicarbonate, 0.002 per cent sodium azide, pH 9.2) (Appendix 4). The tube was repeatedly rinsed with buffer. Only enough of the coating buffer as would coat several plates in a reservoir was poured out. The remainder was left in a glass beaker with constant stirring.

Using the multi-channel pipette (Titertek plus, ICN Biomedicals U.K.), 200 µl of the coating material was added to all wells of one plate and the plate was covered using plate sealer (ICN Biomedicals Ltd. U.K.) labelled accordingly. After incubating plates for 2 hours at room temperature, the coated plates were stored at 4 °C overnight or until needed. Plates can be stored for up to 6 months (Thonar, unpubl.). The plate sealer material was checked periodically to ensure it was airtight and to prevent evaporation of the coating buffer from individual wells.

3. DETERMINATION OF THE TITRES OF ANTIBODIES REQUIRED TO OPTIMISE THE KERATAN SULPHATE ASSAY

3.1. Principle

In order to optimise assay conditions, it was necessary to determine the strength or titre of both antibody preparations used in this assay. A simple direct enzyme-linked immunosorbent assay (ELISA) was used. The principle of this test is that the first antibody will bind to KS antigen adsorbed on the microtitre plate, thereby binding some antibody to the plate. Then, following washing of the plate, a second antibody is added, which is covalently bound to peroxidase, directed against the first antibody. The amount of second antibody bound to the plate is quantified by monitoring the amount of coloured end product formed during final incubation of the substrate for peroxidase.

The objective of the checkerboard design was to test both variables (first and second antibodies) in order to find a point at which both would give the optimum O.D., i.e. between 1.5 and 1.8 absorbance units. The optimum concentration of monoclonal anti-KS antibody (first antibody) was chosen by selecting the second antibody concentration that gave a steep inhibition curve and a near maximum colour development (1.8 absorbance units) after 15 to 30 mins. At that concentration of second antibody, the concentration of first antibody that gave near maximum colour development (i.e. 1.5 to 1.8 absorbance units) was selected.

3.2. Assay method

The anti-KS monoclonal antibody (1/20/5D4, ICN Biomedicals U.K. Ltd.) (first antibody) was prepared as a 1/100 dilution in PBS Tween 20 (sodium chloride 120 mM, disodium hydrogen orthophosphate 5 mM, EDTA 10 mM, Trizma base, 0.5ml, pH 7.0). (Appendix 5).

The first antibody (1/100 dilution) was made up to a 1/1000 dilution in PBS Tween 20, 1 per cent bovine serum albumin (BSA), pH 5.3(antibody buffer) (Appendix 9). The serial half dilutions were prepared in a microtitre plate (transfer plate), (A/S Nunc Denmark), by adding 280 µl of the first dilution, (1/1,000), to the first well and adding 140 µl of antibody buffer to the remaining wells. The first dilution was then serially half diluted. The plate was mixed with shaking for 1 hour at room temperature.

Following mixing, 200 µl of this antibody dilution was transferred to a washed plate, coated with KS antigen and incubated with shaking for an hour at room temperature. The enzyme-labelled antibody (sheep anti-mouse IgG horseradish peroxidase, SAM-HRP, SAPU Ltd. U.K.) (second antibody) titre was determined in the same plate by by testing a narrower concentration range, (i.e. 1/50 to 1/200) of the second antibody (Appendix 6).

3.3. Determination of the titre of first antibody, all other variables known

As it was necessary to acquire a new batch of first antibody during the course of the ELISA work, the optimum concentration of the new batch of monoclonal antibody had to be determined to satisfy the assay requirements.

3.4. Methods

140 µl of PBS-Tween 20, pH 5.3, (instead of sample containing an unknown amount of KS) and 140 µl of first antibody (1/1,000 starting dilution) were added to two wells of a transfer plate. A serial half dilution of this "first" dilution was carried out to a final dilution of 1/128,000 (Appendix 7). The first antibody was pre-incubated for one hour to mimic the actual assay conditions. This mixture contained zero concentration of KS and 140 µl of MAb. The rest of the ELISA was carried out as described above to determine the concentration of MAb required to give an OD between 1.5 and 1.8 at 490 nm.

4. STANDARDISING THE KS ASSAY.

4.1. Materials and methods

International reference standards (IRS) of purified costal cartilage KS were a gift from Dr. A.L. Horwitz, University of Chicago. The IRS contained 411 ng/ml of pure KS. It was necessary to determine the concentration of KS equivalents in our working standard (WS) material, purified at Glasgow Royal Infirmary. The WS was prepared for estimation of its concentration of KS equivalents by making a 1/67 dilution with distilled deionised water containing 0.05 per cent Tween 20 (total volume 25 ml). This was known as WS stock solution. By a process of trial and error, an initial starting dilution of the WS stock solution of 1/25 was chosen. The ELISA assay with an inhibition step was carried out as described earlier and each sample of WS was assayed in duplicate. The results of standardisation of the WS are shown in Table 4.

Starting dilution of working standard	Result (ng/ml of KS equivalents)
1/25	436
1/25	428
1/25	439
1/25	430
1/25	426
1/25	431
Mean value	**432 ng/ml** **SD ± 4.9**

Table 4
Standardisation of the working standard stock (WS) solution.
Results are expressed as ng/ml of KS equivalents.

5. TECHNIQUE OF THE KS ELISA (INCORPORATING AN INHIBITION STEP)

5. 1. Principle of the ELISA technique

Enzyme-linked immunosorbent assays are based on the sandwich technique and have been used widely to determine titres and specificity of antisera as well as to measure antigens both qualititively and quantitatively. The KS ELISA described below incorporates an inhibition step, i.e. the unknown sample and the first antibody, (1/20/5D4), are incubated together for a period of one hour prior to the addition of the specific antibody to the coated plates. The incorporation of an inhibition step allows the quantitation of antigen present in the unknown samples (Rennard, Kimata, Dusemund, Barrach, Wilczek, Kimura and Hascall 1981).

Following the inhibition step, the competitive indirect ELISA is carried out, using the method of Thonar and others (1985) with minor modifications (Thonar and others 1991). The ELISA is represented in diagramatic form in Figure 7. The inhibition mixture, consisting of unknown antigen and first antibody is transferred to washed plates, coated with KS antigen. Any unbound first antibody will bind to coated KS antigen on the plate. After the end of the incubation period, the plate is washed three times. The plate is washed between incubations to remove any antibodies not specifically bound.

A second antibody, SAM-HRP is added. The first and second antibodies are made up in solutions of pH 5.3 (Thonar and others 1991b). This facilitates the quantification of KS, as ELISAs carried out at this pH yield steeper inhibition curves for both standards and unknowns, thereby increasing the ability to discriminate between concentrations of antigen that are not markedly different. The lower pH also helps to reduce non-specific binding. The plate is washed again following incubation.

The amount of second antibody bound to the plate is quantitated by monitoring the amount of coloured end product formed during the final incubation with substrate for the peroxidase. The concentration of KS antigen present in the unknown sample is calculated by comparing the absorbance value in each case to values generated from known concentrations of highly purified human costal cartilage KS antigen equivalents, used as WS in the assay.

The intensity of the colour reaction is inversely proportional to the amount of antigenic KS in the sample. The WS is treated in an identical manner to the unknown samples and is incorporated in the same plate. All concentrations of synovial fluid and serum KS reported in this study represent equivalents of this WS. Half-inhibition binding of the antibody to the coated antigen occurs when the antigen is present at a concentration equivalent to 5 ng of WS per ml of solution tested. Complete inhibition (>95 per cent) was reached at a concentration of 15 ng per ml. This is defined as the working range of the assay under the conditions described below.

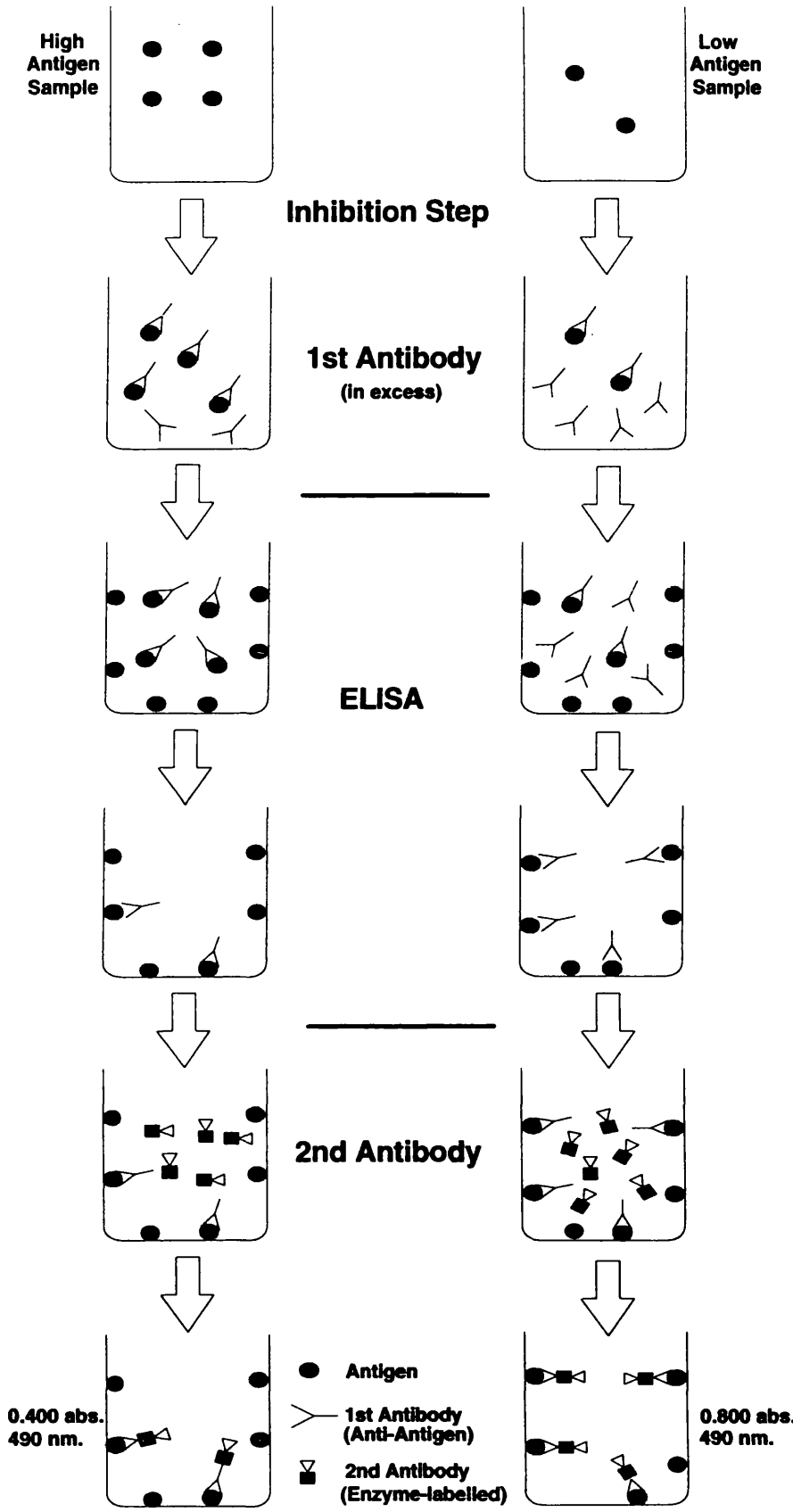

Figure 7
Principle of the KS ELISA incorporating an inhibition step

5.2. Collection and processing of samples

Blood samples (7 to 10 ml) were collected aseptically by jugular venepuncture into vacutainer tubes containing no anticoagulant. Synovial fluid samples were aspirated aseptically (without opening the joints in normal animals in Experiment 1) using a 19 gauge 1 ½ inch needle and syringe. All samples were stored at 4 °C until processing within three hours of collection. Both synovial fluid and blood samples were centrifuged at 4 °C at 2054 *g* for 10 mins. Serum was separated and stored at —20 °C in 1.5 ml Treff tubes (Scotlabs. U.K.) The synovial fluid aspirate was stored under the same conditions following centrifugation. All synovial fluid and serum samples were identified according to the **Joint Naming Code** (Appendix 8).

5.3. Inhibition step

Details of the composition of buffers used in the assay are contained in Appendices 5 and 9. The WS and the unknown serum and synovial fluid samples were diluted in working buffer in 3 ml polypropylene tubes (Sarstedt UK).The working standards were diluted 1/4 as the starting dilution. They were stored at —20 °C until required and once in use, were stored at 4 °C for 2 to 3 days.

The "unknown" equine serum and synovial fluid samples were stored at —20 °C and once thawed, were stored at 4 °C until used. Prior to dilution, all samples were mixed thoroughly (Maxi-Mix 1, Radley's Lab. Equipt. U.K.) for 10 seconds. Once diluted in working buffer, all samples were mixed thoroughly again for 10 seconds immediately prior to pipetting. In general, serum was diluted either one in five or one in ten with working buffer as the starting dilution, and synovial fluid was usually diluted 1/100 or 1/200 as the starting dilution.

Using the plate template as a guide, 280 µl of each first dilution sample and standard was pipetted into the appropriate well. A multichannel pipette (Titertek Plus, ICN Biomedicals Ltd. U.K.) was used to add 140 µl of working buffer to all remaining wells. Using the multichannel pipette, serial half dilutions were performed. All wells finally contained 140 µl of sample and PBS.

The appropriate concentration of first antibody was diluted in antibody buffer. The concentration of anti-KS monoclonal antibody used varied from one batch of plates to another, depending on the half-inhibition value of the batch. (The first two batches of plates required a concentration of 1/30,000 MAb. The third batch of plates required a MAb concentration of 1/25,000). Following thorough mixing, and using the multichannel pipette, 140 µl of the MAb dilution was pipetted into each well of the microtitre plate. The plate was incubated with shaking on the Varishaker for 1 hr at 25 °C and the ELISA carried out, or the plate was covered with a plate sealer (ICN Biomedicals Ltd. U.K.) and stored until the next day for assay.

5.4. The ELISA technique

The inhibition mixture of first antibody with either standard or sample was incubated in coated plates. After washing, an enzyme-labelled antibody (second antibody) directed against the first antibody was pipetted. Finally the substrate for the enzyme was added and colour development occurred.

The coated plates, (stored at 4 °C) were allowed to come to room temperature (if left overnight). The coating buffer was removed from the plate and three five minute washes with working buffer were carried out. Using the multichannel pipette, 200 µl of the inhibition mixture in the transfer plate was transferred to the washed coated plate using fresh pipette tips for each row of wells. The coated plate was covered and incubated for 1 hr at room temperature with shaking.

Following incubation, the inhibition mixture was discarded and plates were washed again three times using working buffer. Ten minutes prior to use, the second antibody was prepared in antibody buffer at the appropriate dilution, e.g. 1/125. Using the multichannel pipette with clean tips, 200 µl of diluted SAM-HRP was pipetted into the washed coated plate, covered with a plastic plate lid and incubated at room temperature with shaking for 1 hr.

After 45 mins. of incubation of the coated plate with the second antibody, the substrate for the colour reaction was prepared and tested. O-phenylene diamine (OPD) colour reagent was prepared (25 ml of 0.05M citric acid, 0.10M disodium hydrogen orthophosphate, 10 mg of OPD, 11 µl of hydrogen peroxide, 30 per cent w/v, Appendix 9). The second antibody was removed from the coated plate and three five minute washes were carried out with working buffer. Using the multichannel pipette with clean tips, 200 µl of prepared substrate was added to the washed coated plate and the colour development carefully monitored over the ensuing 15 to 30 min. period (Figure 8).

The plate was read at approximately 4 min intervals on a microplate photometer (Dynatech Laboratories Ltd., U.K.) at 490 nm until the optimum assay conditions had been reached, i.e. the standards gave an O.D. reading between 1.5 and 1.8 at 490 nm. The half inhibition of the standard curve of the colour reaction occurred when the antigen was present at a concentration equivalent to 5 ng of working standard KS per ml of solution to be tested (Figure 9). Complete inhibition (> 95 per cent) occurred at a concentration of 15 ng per ml.

The calculation of the amount of KS equivalents in the "unknown" samples was derived using Dynatech software (Dynatech MR 5000, Dynatech Labs. Ltd., UK). Only values which fitted on the linear portion of the semi-log curve were accepted. Values of the unknown samples were calculated from the average of the absorbances obtained on duplicate samples which were referenced against the WS incorporated into each plate. Values fitting on the linear portion of the standard curve between a 30 per cent to 60 per cent inhibition window were accepted. If more than one value from the same sample fitted on the curve then

the values were averaged and this figure taken to represent the true value measured. Where either serum or synovial fluid dilutions were inappropriate such that no values fitted on the curve, the starting dilution was adjusted and the sample repeated.

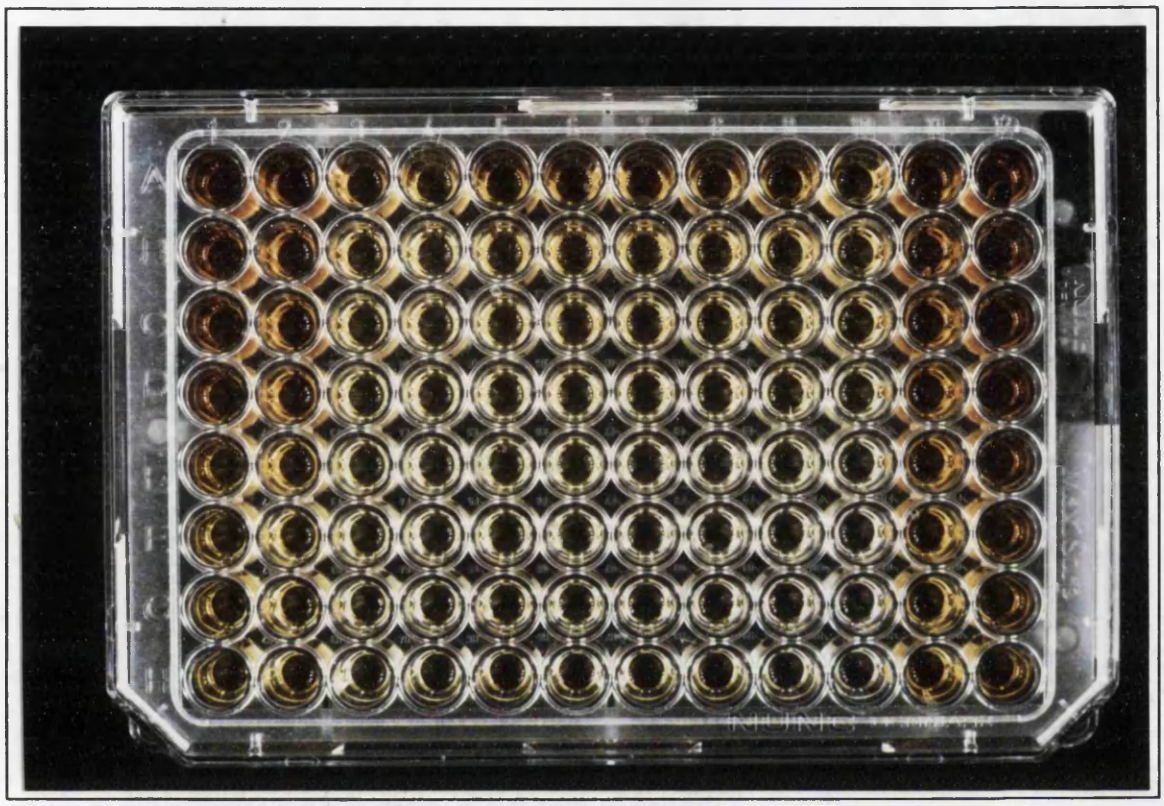

Figure 8. Colour development in the keratan sulphate ELISA.
The intensity of the colour reaction is inversely proportional to the amount of keratan sulphate in the sample.

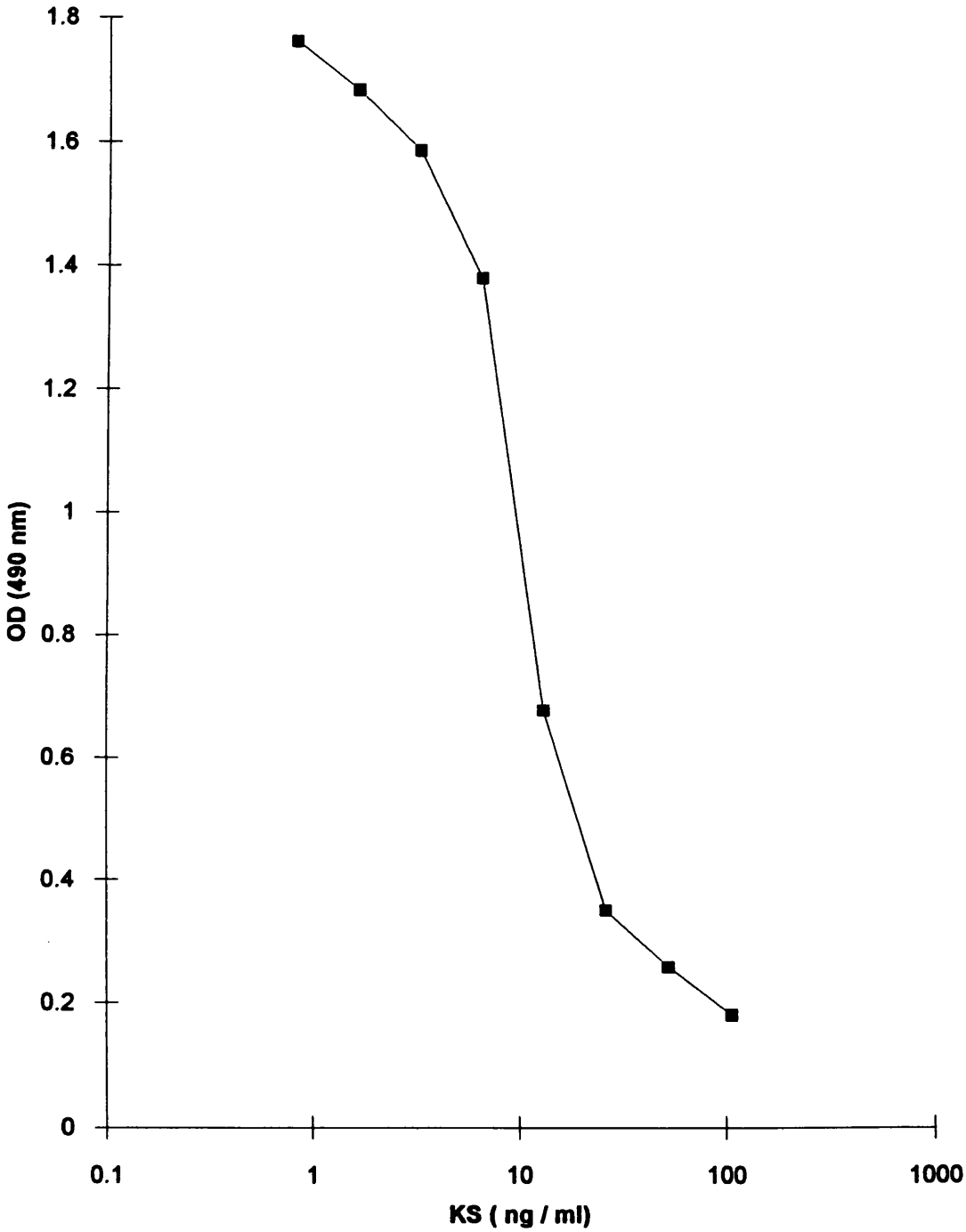

Figure 9. KS standard curve

6. PROCEDURES FOR VALIDATION OF THE KS ELISA

6.1. Intra-assay Coefficient of Variation

An intra-assay coefficient of variation (C.V.) test was carried out using serum at a starting dilution of 1/10 in working buffer (Appendix 10). Each serum sample was serially half-diluted from a starting dilution of 1/10. The plate template was designed as in Appendix 10. The ELISA with an inhibition step was carried out under identical conditions as with a routine assay. The values obtained from the test are shown in Table 5. The intra-assay C.V. was calculated at 4.3%.

6.2. Interassay Coefficient of Variation

An interassay C.V. was established for each batch of plates. A quality control serum sample was incorporated into each plate. A paired t-test was carried out on the values obtained with the quality control serum included in each plate and assayed in each batch. The inter-assay C.V.on the first batch of plates was 11.5 per cent, the second batch of plates had an inter-assay C.V. of 10.5 per cent and the third was 10.9 per cent (Table 6). The first two batches of plates required a first antibody concentration of 1/30,000 and the third batch of plates required a first antibody concentration of 1/25,000. Plates giving results within the percentage range calculated on the batch of plates being used were accepted as valid. Results outwith this percentage range were excluded.

6.3. Effect of storage on samples

A serum sample, initially diluted one in five with working buffer was stored at —20 °C, 4 °C and at room temperature to evaluate the effect, if any, of different storage conditions on the stability of the antigenic KS epitopes in the samples. Prior to storage over an eight week period, the concentration of KS was estimated. The samples were stored for this length of time as all serum and synovial fluid samples collected were assayed for KS within two months of collection and processing for storage. The results of this experiment are shown in Table 7.

6.4. Spiking the assay

In order to test the accuracy of the assay, and the possibility of interference, i.e. blocking factors in serum, a serum sample containing a known amount of KS (previously assayed twice consecutively) was spiked with known concentrations of WS.

The WS contains 433 ng/ml of purified KS. The serum sample contained 118 ng/ml of KS equivalents. A 1/5 starting dilution of the serum sample without a spike in working buffer was incorporated into the plate. The first spike contained 54 ng/ml of WS. 125 µl of WS containing 54 ng/ml was added to 100 µl of 1/5 diluted serum. A second spike was prepared to contain 108 ng/ml of WS. 125 µl of this 1/4 dilution was added to 100 µl of 1/5 diluted serum. Samples were prepared and assayed in duplicate using the competitive indirect ELISA with an inhibition step as described above.

From the results of the spike assay, (Table 8) it can be seen that there was a linear increase in response on addition of exogenous KS to serum of known KS concentration. The first spike of 54 ng/ml of WS gave a reading that was 15 per cent above the predicted value. This was outwith the 10.9 per cent interassay C.V.

The second spike of 108 ng/ml gave a reading 35 per cent greater than the predicted value. This was higher than the 10.9 per cent variation allowed on the interassay C.V. Although this reading was high, it showed that there were no blocking factors reducing the measurement of KS in equine serum, a finding that agreed with the work of Seibel, Towbin, Braun, Kiefer, Muller and Paullsen (1989) using the ELISA assay on human serum.

Well No.	**KS equivalents.**
3 and 4	578, 536
5 and 6	571, 522
7 and 8	542, 585
9 and 10	522, 574
11 and 12	572, 531
Mean ± S.D. **Intra-assay C.V.**	**554 ± 24** **4.3%**

Table 5
Intra-assay coefficient of variation (C.V.)
The quality control (QCS) serum values are expressed as ng/ml of KS equivalents. The QC serum was diluted 1/10 as a starting dilution in working buffer and serially half-diluted subsequently. The C.V. is calculated as SD divided by the mean value.

Plate Batch no.	**Mean**	**Median**	**S.D.**	**S.E.M.**	**C.V.**	**N**
1	523.4	521	60.7	21.5	11.5%	8
2	523.6	514	55.2	13.4	10.5%	17
3	460.4	441	50.3	15.2	10.9%	11

Table 6
Inter-assay coefficient of variation.
S.D.= standard deviation, S.E.M.= standard error of the mean, C.V.= coefficient of variation, calculated as S.D.divided by the mean. Quality control serum (QCS) was incorporated in each plate assayed. The QCS values are expressed as ng/ml of KS equivalents.

Storage conditions	KS equivalent value.
Original sample	162
Minus 20 °C	118
Room Temperature	***
4 °C	67.7

Table 7
Effect of storage (8 weeks) on the measurement of KS in serum.
All results are expressed as KS equivalents in ng/ml.
***= sample deteriorated and contaminated with fungal growth, thus, no value obtained.

Sample	Calculated Result	Actual Result	% of calculated result
serum x	118		
x + spike 1	172	197	115
x + spike 2	226	306	135

Table 8
Spiking the ELISA with working standard (WS).
KS results are expressed as ng/ml.
Total volume of sample x plus spike= 500ul
Both spike samples are added to 1/5 starting dilutions of serum x.
Percentage values are expressed as absolute values.
Spike 1 = 54.1 ng/ml of WS (1/4 diln.)
Spike 2 = 108 ng/ml of WS (1/8 diln.)

6.5 Curve fitting procedures

In order to ascertain if the standard curve and the equine sample curves were parallel, the optical density values for six randomly selected equine serum samples were plotted against their dilution values (represented as a negative log scale) (Figure 10). From graphical evidence, the equine serum samples did not appear to be parallel to the standard curve. This suggested that there was an unknown factor/factors in equine serum which interfered with the assay in terms of the OD reading obtained.

The equine serum sample curves shown in Figure 10 were modelled using the approach of De Lean, Munson and Rodbard (1979). There was strong statistical evidence of difference in slope between the standard curve and some of the samples. When e.g., Horse 1 was compared with the Standard curve, the t statistic for comparing the slope parameters was 3.92 with 6 degrees of freedom ($p = 0.008$). However, in terms of estimating the difference in log concentration between the standard and the sample, it made little difference in this instance whether parallel or non-parallel lines were fitted to the data (0.7, standard error 0.12, versus 0.78 with a standard error of 0.05 for parallel and non-parallel lines respectively (G Murray, personal communication).

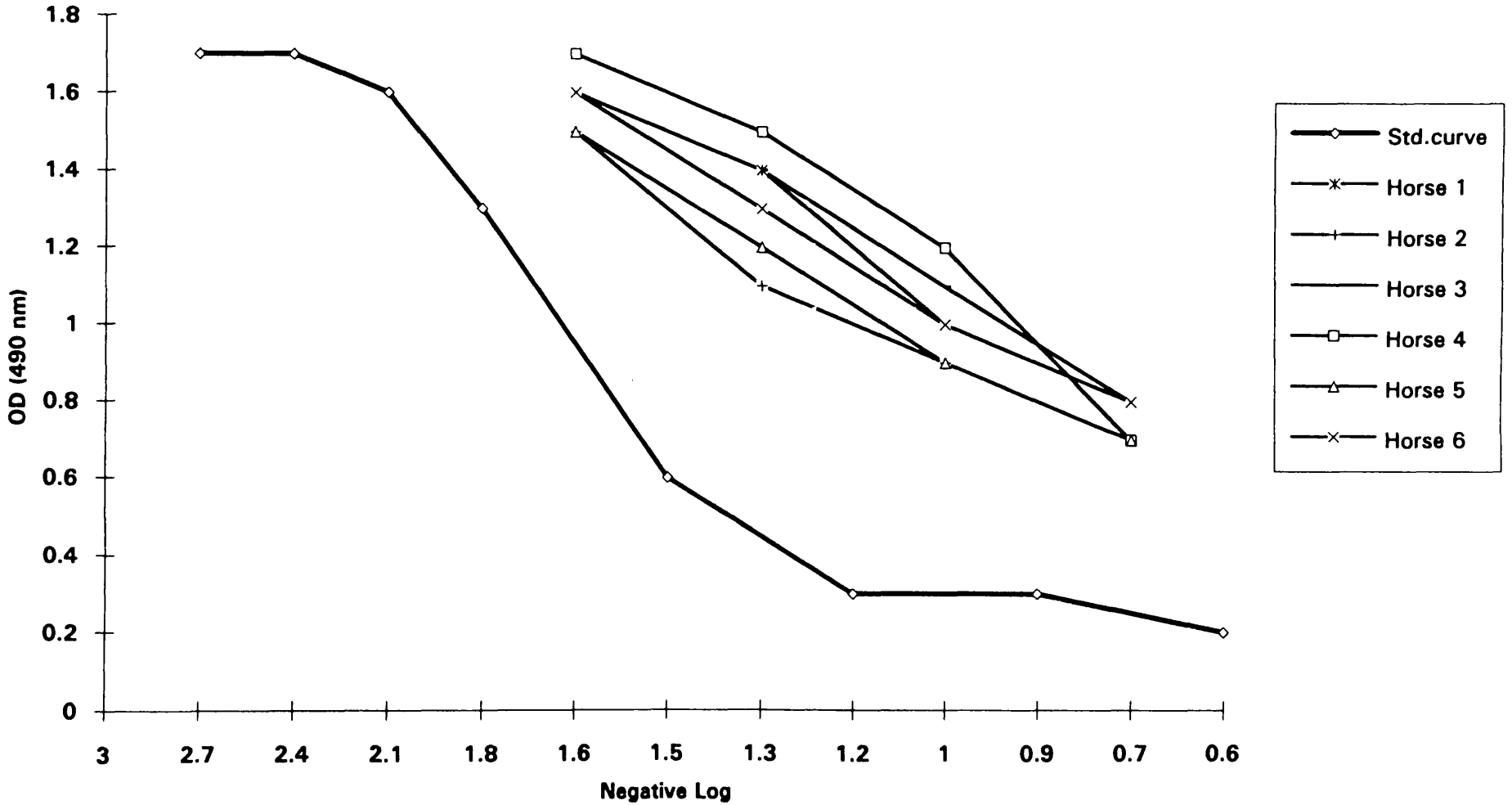

Figure 10
KS standard curve and equine serum sample dilutions showing non-parallelism

6.6. Cross Laboratory Quality Control

As part of a quality control exercise, four samples, serum and synovial fluid, human and equine) were assayed in two consecutive assays at Glasgow Royal Infirmary (GRI). The same samples were analysed at Rush-Presbyterian-St. Lukes Medical Centre, (RP) Chicago, under similar laboratory conditions using the same assay for KS. The interassay C.V. at GRI was 10.9 per cent. The interassay C.V. at RP was 11 per cent (Thonar, 1993 unpublished).

The mean value (calculated from two consecutive measurements on each sample, assayed in duplicate) is shown in Table 9. The absolute difference and percentage difference between samples is shown. The percentage difference is taken as the percentage change in the value obtained at RP as compared to GRI (taken as 100 per cent). Results were analysed using Students paired t-test, a level of significance $p<0.05$, 95 per cent confidence interval [CI]. Although the sample numbers are small, (n=4), there was no significant difference in the results obtained in both laboratories.

Sample	GRI	RP	Difference (abs.) value and % value
ESE	523	545	22 104 %
ESF	6588	4642	1946 70 %
HSF	23687	24190	503 102 %
HSE	387	474	87 122 %

Table 9
Quality control on samples assayed in two different laboratories
ESF= equine synovial fluid, **ESE** = equine serum. **HSF**= Human synovial fluid, **HSE**= Human serum. **GRI**= Glasgow Royal Infirmary, **RP**= Rush Presbyterian.

CHAPTER III

STUDIES IN NORMAL HORSES

1. EXPERIMENT 1. OBJECTIVES

The objective in evaluating KS levels in the joints sampled in a group of "normal" horses was to obtain a range of values for the individual joints in order to assess:

(A) If there was any difference in values obtained from right and left paired joints between animals.
(B) If there was any correlation between serum and joint levels between animals.

In the group of animals sampled, it was decided to compare joints as follows: Paired, (i.e. right and left) radiocarpal joints were compared between animals. Paired midcarpal joints were compared between animals. Left radiocarpal and midcarpal joints were compared with right radiocarpal and midcarpal joints between animals.

Each of the carpal joints sampled was compared with the tibiotarsal joint on the corresponding side i.e. right radiocarpal compared to right tibiotarsal joint between animals and right midcarpal compared to right tibiotarsal joint between animals. The same procedure was carried out for left-sided joints. Serum KS values were compared with joint values (all joints) to assess if there was any correlation between serum and synovial fluid KS levels.

2. SUBJECTS, SAMPLES AND METHODS

KS levels were evaluated in a group of "normal" horses, i.e. defined within the context of the current study as animals that had no evidence of or history of joint disease, that were euthanased for other reasons (Appendix 11). The horses ranged in age from 2 years old to 15 years old. Seven horses were Thoroughbred or Thorougbred-cross, two were Clydesdales and two were ponies. Seven geldings and four mares were used. All animals had no evidence of articular cartilage pathology on visual inspection at at post-mortem examination.

Serum samples were collected by jugular venepuncture (7 to10 ml), prior to death in most cases. Synovial fluid was collected immediately post-euthanasia by needle aspiration, using a 19 gauge 1 ½ inch needle and syringe without opening the joint. Subsequent to needle aspiration, each joint was opened and the articular cartilage visually inspected. Synovial fluid collected was processed (within one hour) as described on page 42 and stored at —20 °C until assayed.

2.1. Sites for synovial fluid aspiration

Synovial fluid was aspirated from the radiocarpal joint and from the midcarpal joint (Figure 12). The latter joint communicates with the carpometacarpal joint.

With the carpus flexed, the radiocarpal joint was located and the sample collected from a site midway between the medial edge of the radius and the proximal edge of the radial carpal bone.The needle was inserted at this site, medial to the medial edge of the extensor carpi radialis tendon. The volume of synovial fluid recovered was on average, 3 to 8 ml.

The midcarpal joint was located and the needle inserted medial to the extensor carpi radialis tendon. The average volume of fluid collected was 3 to 6 ml.

The tibiotarsal joint (Figure 11) was penetrated on the medial side of the saphenous vein as it traverses the joint vertically, approximately 2 to 3 cm below the level of the medial malleolus at the distal end of the tibia. The average volume collected was 5 ml.

The synovial fluid collected was assessed visually for any abnormalities, e.g. colour change or presence of debris. In all cases, synovial fluid appearance corresponded to what has been described as normal (McIlwraith 1987).

2.2. Statistical methods

Results were analysed throughout this thesis using the Minitab Release 9 statistical package (Appendix 1). The values for each joint in all animals and the corresponding serum values are contained in Appendix 12.
There was a wide range of values for each joint in the group of animals studied (Table 11 and Appendix 11). The values were checked and did not appear to be normally distributed. Due to the non-parametric nature of these values, ratios for paired values, rather than absolute values were used to normalise the data for statistical analysis.

Ratios were calculated for paired joints, e.g. BR/BL in all animals. The mean ratio across animals was then calculated. The ratios were plotted as histograms and found to be approximately normally distributed. Students t-test was then used on the mean ratios to test the null hypothesis that the mean ratio was equal to one, versus the alternative hypothesis that the mean ratio was not equal to one.

A level of significance, $p < 0.05$ was used. The values for the paired joint ratios 95% confidence interval [CI], and significance levels are shown in Table 10. The median and range of synovial fluid KS values and serum values are shown in Tables 11 and 12 respectively.

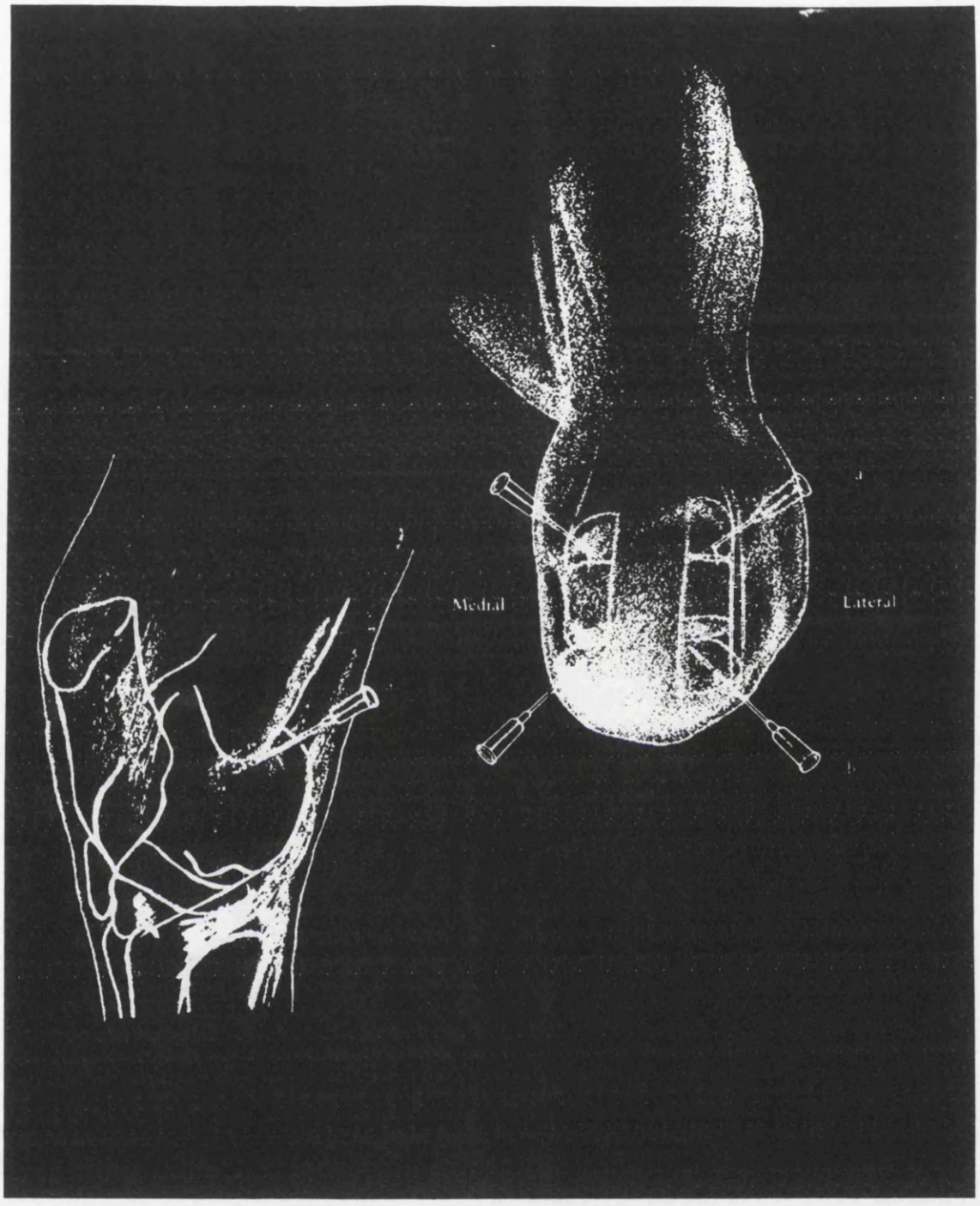

Figure 11
Plantaromedial view of the tarsus.
(a) Site of sampling the tibiotarsal joint.

Figure 12
Dorsal view of the flexed carpus.
(a) Site of sampling from the radiocarpal joint.
(b) Site of sampling from the midcarpal joint.

2.3. Results

Paired joint analysis (Table 10)

The individual joint values are represented for each animal as well as the median value for each joint in all 11 horses in Figures 13 to 18.

There was no significant difference in right and left radiocarpal joints between the 11 animals. There was no significant difference in right and left midcarpal joints between the 11 animals. Right and left tibiotarsal joints were compared between animals, (n=11). Again, there was no significant difference in the values obtained.

The mean values obtained for the left carpal joints, i.e. the sum of the left radiocarpal and left midcarpal joints was compared to the sum of the right carpal joints as a ratio. There was no significant difference between the sum of the right carpal joint values and the left carpal joint values. The level of KS in the left radiocarpal joint was significantly higher than in the left midcarpal joint in all animals, p= 0.041, 95% CI [1.015, -1.631]. The left radiocarpal joint was compared to the left tibiotarsal joint. Again there was no significant difference in the values obtained.

Each carpal joint value was compared as a ratio to the tibiotarsal joint on the corresponding side in all animals i.e. right radiocarpal joint versus right tibiotarsal joint, right midcarpal joint versus right tibiotarsal joint etc. Again, there was no significant difference between these paired fore and hind limb joints. There was a broad range of serum keratan sulphate values in the group of 11 horses, (median 183 ng/ml, range 82-428 ng/ml), (Table 12).

Serum and the mean value of all joint values for each animal (Appendix 12) were compared to assess if there was any correlation between serum and synovial fluid in all animals. There was no correlation, (Figure 19), a finding that is in agreement with the results of other authors (Alwan and others 1990).

In conclusion, results obtained in this study of 11 animals show that there is a wide range in KS values between animals in the joints sampled. There does not appear to be any correlation between serum and synovial fluid KS levels measured in these horses. There appears to be no difference in KS values in paired carpal and tarsal joints between animals. There was a significant difference in the values obtained for the left radiocarpal joint compared to the left midcarpal joint between animals, in that KS levels were higher in this joint compared to the midcarpal joint in all animals. Horses No. 2, 8, 3 and 11 had values for the left radiocarpal joint that were more than twice the value of the corresponding midcarpal joint (Appendix 12). Grossly, these joints had been visually inspected and therefore, in these animals, the greater value cannot be explained on the basis of articular cartilage pathology or abnormal loading and wear on the joints.

JOINT RATIO	MEAN RATIO	95% CONFIDENCE INTERVAL	Level of signific.
BR/BL	0.92	0.57, 1.27	NS
CR/CL	1.22	0.86, 1.56	NS
ER/EL	0.99	0.83, 1.14	NS
$\frac{mBR+CR}{mBL+CL}$	1.03	0.68, 1.38	NS
$\frac{mBR+BL}{mCR+CL}$	1.11	0.91, 1.31	NS
BR/CR	0.91	0.72, 1.10	NS
BL/CL	1.32	1.02, 1.63	p=0.041
BR/ER	1.05	0.74, 1.35	NS
CR/ER	1.17	0.88, 1.45	NS
BL/EL	1.26	0.97, 1.55	NS
CL/EL	1.05	0.65, 1.45	NS

Table 10
Normal equine paired joint ratios, (n=11).
m = mean value. B= radiocarpal joint, C= midcarpal joint, E= tibiotarsal joint, R=right, L= left.
NS= non-significant.

JOINT	MEDIAN	RANGE
BR	36,400	5,300 - 204,474
BL	49,293	5,086 - 193,043
CR	40,962	6,820 - 185,139
CL	38,870	6,293 - 267,216
ER	57,619	6,631 - 99,776
EL	59,270	7,163 - 144,333

Table 11
Median and range of KS values for individual joints in normal horses (n=11).
B= radiocarpal joint, C= midcarpal joint, E= tibiotarsal joint.
R=right, L= left. Results are expressed as KS equivalents (ng/ml).

	MEDIAN	RANGE	MEAN ± SD
Serum	183	82 - 428	215 ± 92

Table 12
Serum KS values for normal horses (n=11).
Results are expressed as KS equivalents (ng/ml).

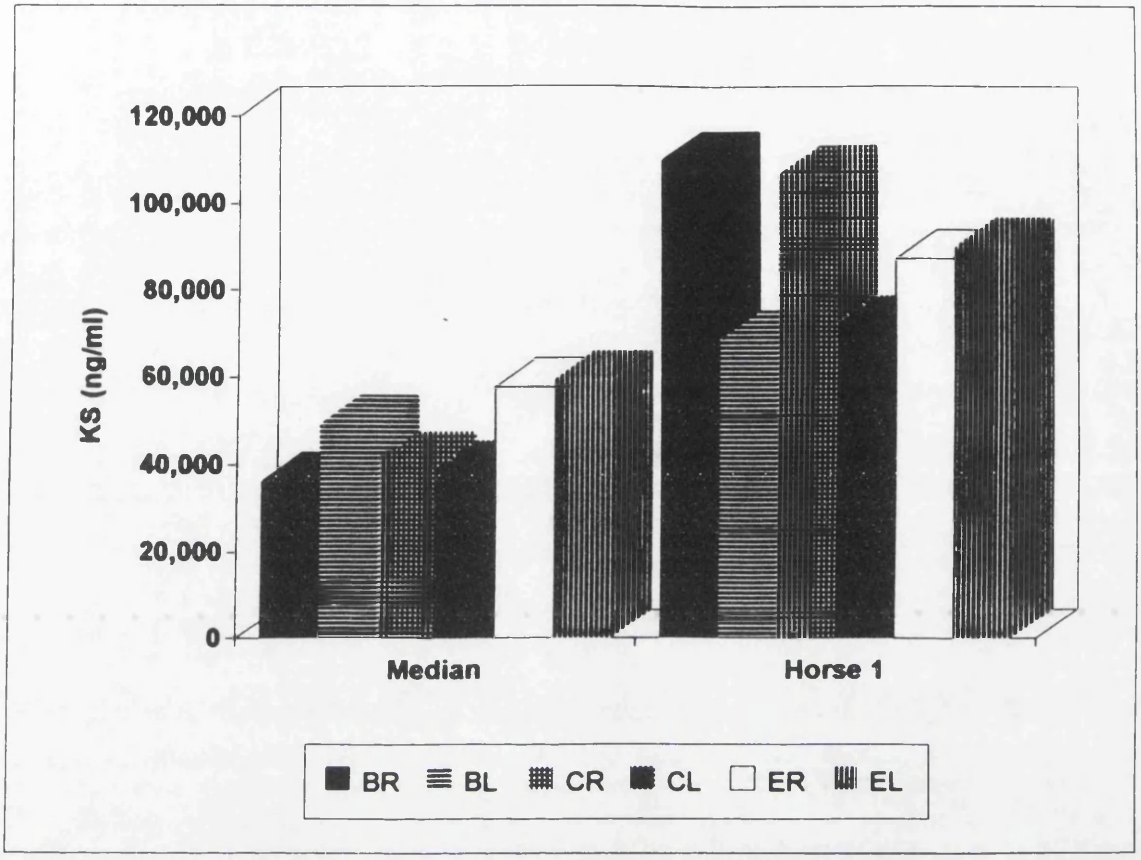

Figure13. Median KS values for each joint (n=11) and KS values for Horse 1

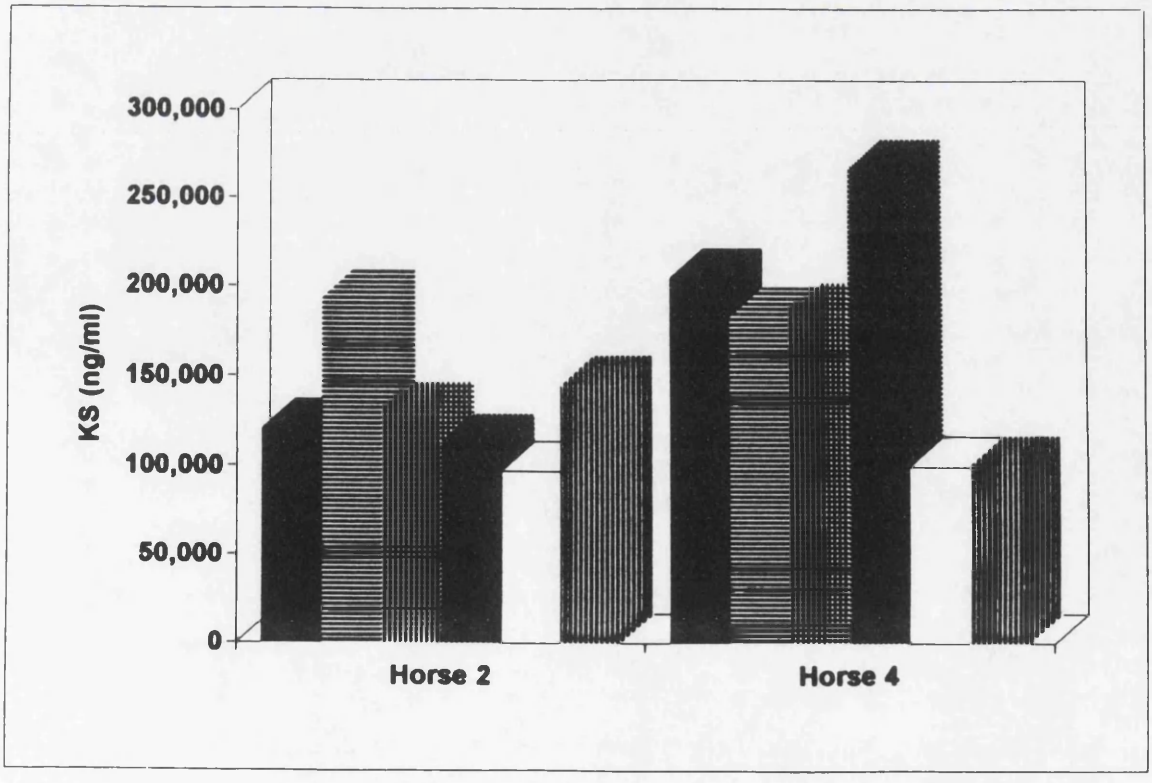

Figure 14. KS values for each joint in Horse 2 and 4

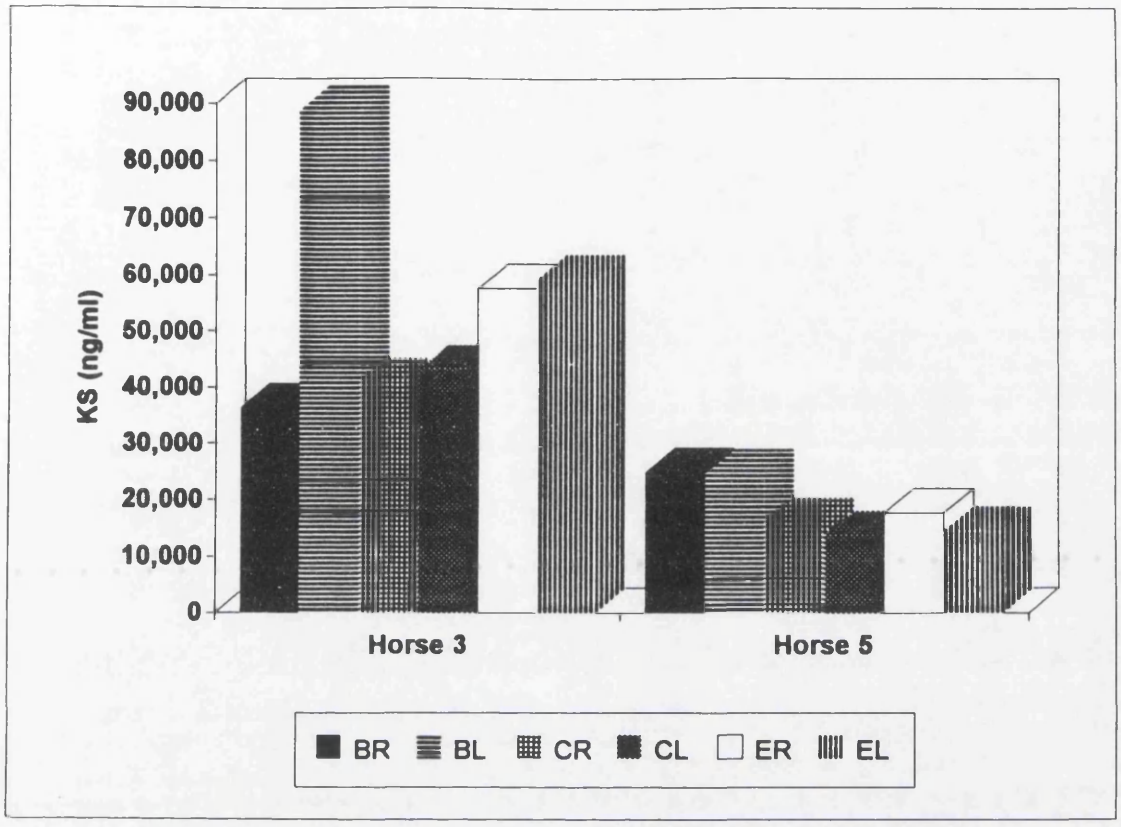

Figure 15. KS values for each joint in Horse 3 and 5

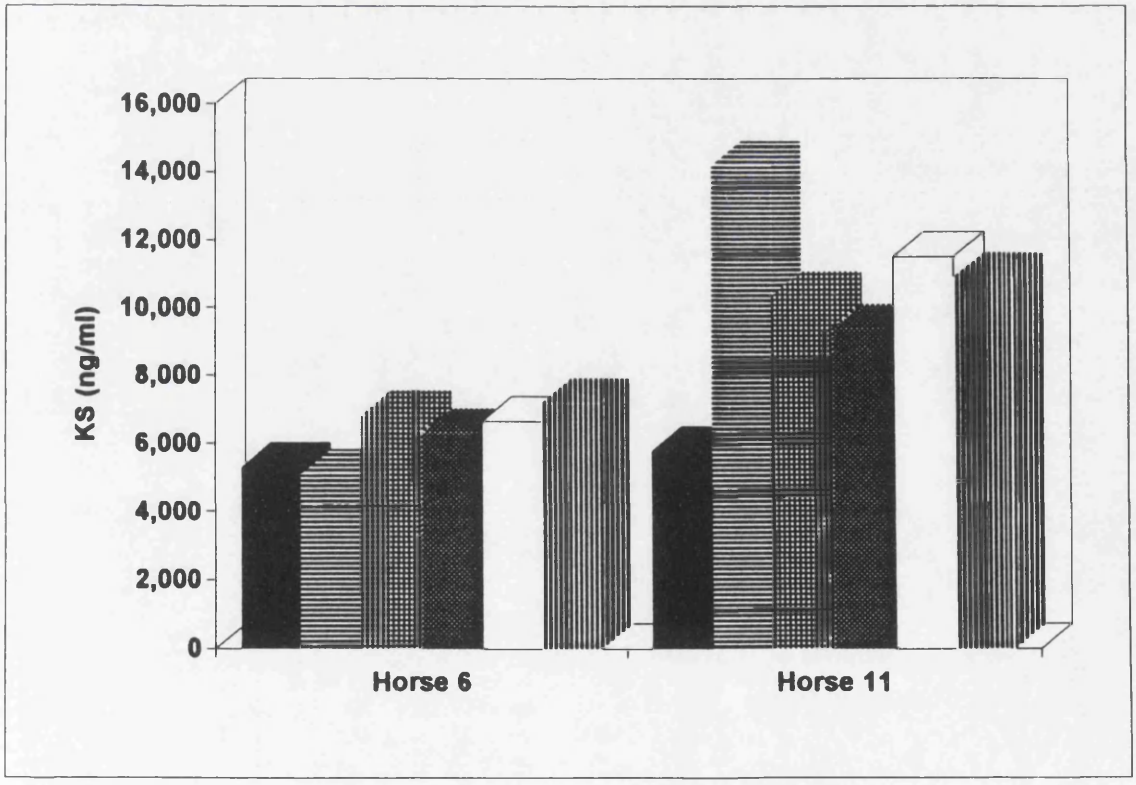

Figure 16. KS values for each joint in Horse 6 and 11

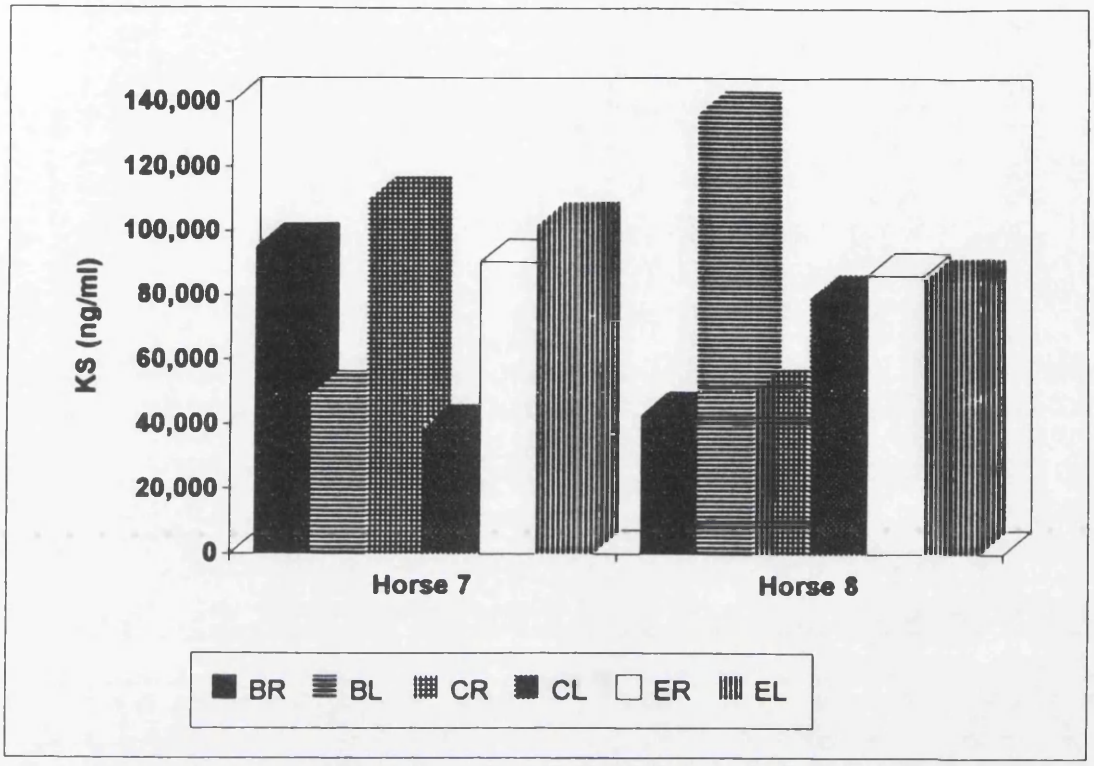

Figure 17. KS values for each joint in Horse 7 and 8

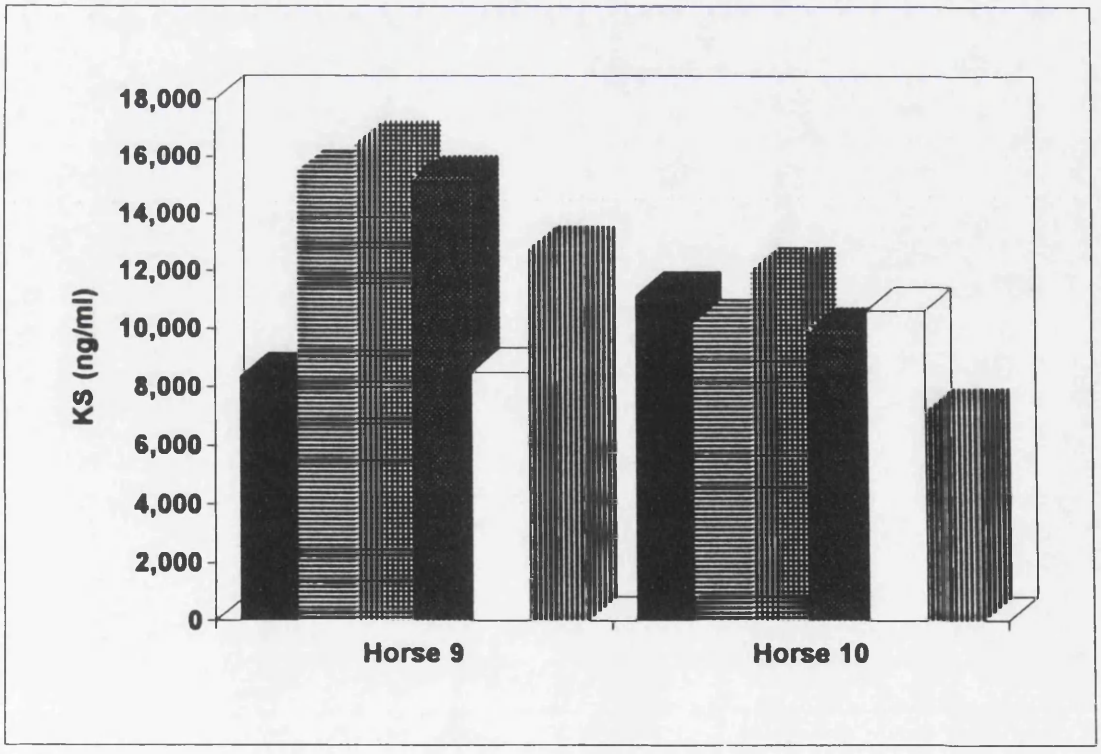

Figure 18. KS values for each joint in Horse 9 and 10

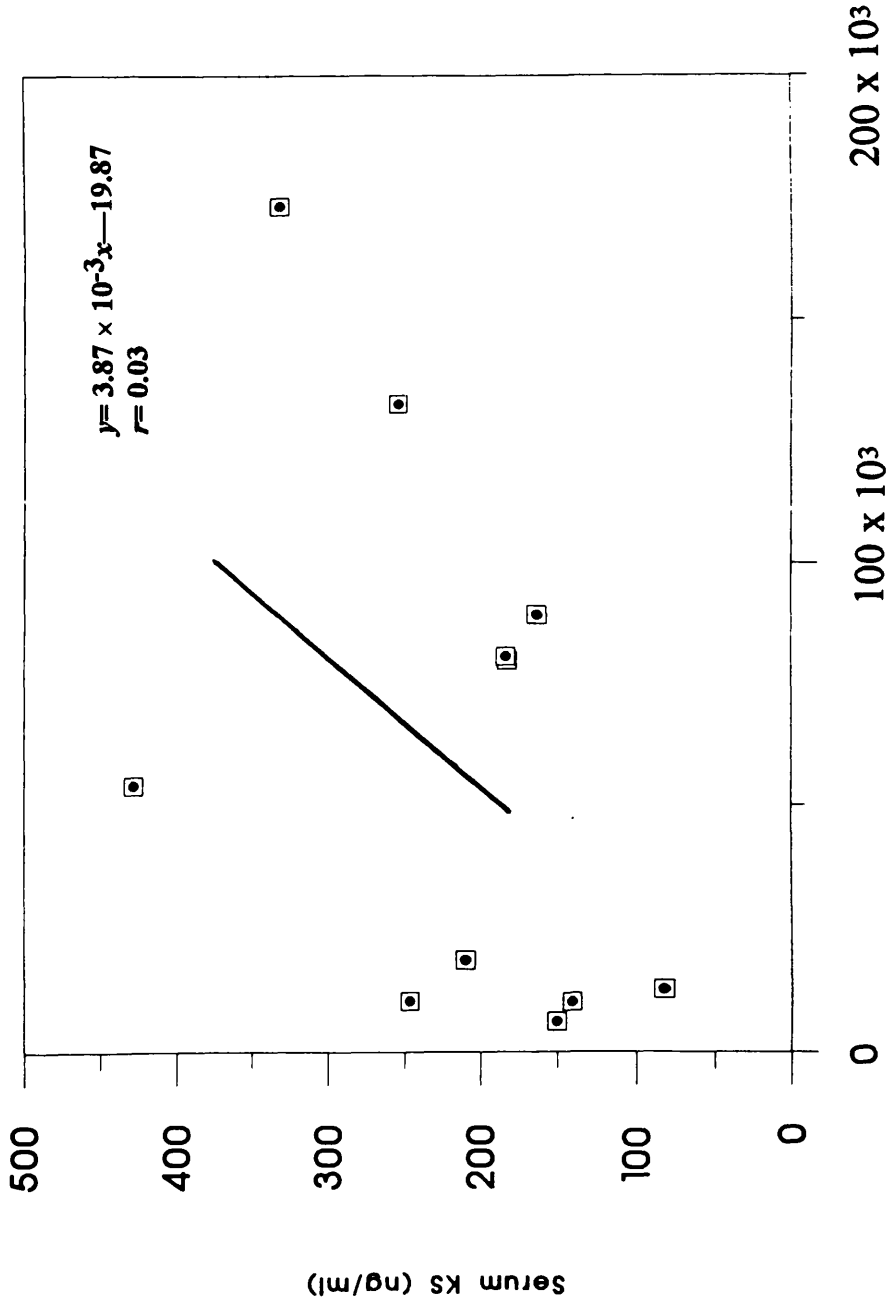

Figure 19. Synovial fluid and serum values: Lack of correlation

3. EXPERIMENT 2. DIURNAL VARIATION IN SERUM KS LEVELS.

OBJECTIVES

In order to investigate whether there is diurnal variation in serum KS levels, six normal ponies, housed with no exercise, (Appendix 13) were sampled at 6 hour intervals (three times) within one day. These animals were defined as normal within the context of this study on the basis of no history of joint disease and no evidence of joint disease on inspection. Four fillies and two colts were used. The ponies were kept stabled with no exercise. The ponies were aged 15 to 16 months. Blood samples were collected by jugular venepuncture (7 ml/sample) and processed as outlined on page 42. The ELISA was carried out as described earlier .

3.1. Results

Serum KS results are shown in Table 13. Results were plotted on a line graph (Figure 20). It was decided to analyse the data non-parametrically as one of the animals, No. 103, has very high serum KS values skewing the data, which did not appear normally distributed. A Kruscall-Wallis test was carried out, with a significance level $p < 0.05$ taken as statistically significant.

From Figure 20, there is graphical evidence that there was an increase in serum KS levels in two of the six animals between 10 am and 4 pm. In three animals, serum KS decreased between 10 am and 4 pm and in one animal there was no change.

Between 4 pm and 10 pm there was a decrease in serum KS as seen graphically in three of six animals. In two of six animals, there was an increase in serum KS and in one animal there was no change evident. The mean serum KS value between 10 am and 4 pm decreased by 6 per cent. Between 4 pm and 10 pm there was a 4 per cent increase in mean serum KS levels.

Statistically, there was no significant difference between values obtained at 10 am and 4 pm and between 4 pm and 10 pm in all six ponies. There was no difference in values obtained at 10 am and 10 pm in all animals. There was a broad range of serum KS values in the six ponies at the various time intervals over the samping period (mean value, 166 ng/ml, range 70 to 451 ng/ml). This has also been observed in human studies investigating diurnal and day to day variation in serum KS levels (Block and others 1989). In conclusion, in the group of six ponies studied, there was no statistical evidence of diurnal variation in serum KS levels.

4. EXPERIMENT 3. DAY TO DAY VARIATION IN SERUM KS LEVELS

OBJECTIVES

Day to day variation in serum KS levels was investigated using a blood sampling protocol similar to that of Block and others (1989). Six ponies (same animals as in Experiment 2) were sampled (7 ml/sample) by jugular venepuncture on three separate occasions at 48 hour intervals. All samples were collected at the same time point (10 am) on each occasion. The animals were kept stabled during this period, with no exercise. Samples were processed as above.

4.1. Results

Due to the presence of very high values in one animal, (No.103), which skewed the data, which did not appear normally distributed, non-parametric tests (Kruscall-Wallis) were carried out. The values obtained for each animal at the various time intervals are shown in Table 14. Values obtained were plotted graphically (Figure 21).

From the values plotted on the line graphs, there appears to be a decrease in serum KS levels in three of the six animals between Day 1 and Day 3. Two of the six animals showed an increase between Day 1 and Day 3, with one animal showing little or no change. Between Day 3 and Day 5, there was an increase in serum KS in four of the six animals, with a decrease in the remaining two animals.

Statistically, however, there was no significant difference between values obtained on Day 1, Day 3 and Day 5. In conclusion, there was no statistically significant evidence of day to day variation in serum KS levels in the group of animals studied.

PONY NO.	10 AM	4 PM	10 PM
101	135	80	147
102	87	122	74
103	450	451	376
104	92	70	71
105	125	73	183
106	129	170	161
MEAN	170	161	169

Table 13
Diurnal variation in serum KS levels
Results in ng/ml of KS equivalents.

PONY NO.	DAY 1	DAY 3	DAY 5
1O1	135	141	168
102	87	99	120
103	450	430	364
104	92	67	136
105	125	127	100
106	129	72	99
MEAN	170	156	165

Table 14
Day to day variation in serum KS levels
Results in ng/ml of KS equivalents.

ANIMAL	MEAN ± S.D.	RANGE	Max.% change from mean
101	148 ± 14.4	135 - 168	13
102	101 ± 15.1	83 - 120	19
103	415 ±36.7	364 - 450	8
104	98 ± 28.5	67 - 136	38
105	117 ± 12.3	100 - 127	9
106	100 ± 23.3	72 - 129	29

Table 15
Day to day variation in serum KS (ng/ml), mean, standard deviation and range.

ANIMAL	MEAN ± S.D.	RANGE	Max.% change from mean
101	121 ± 29.2	80 - 147	21
102	94 ± 20.3	74 - 122	30
103	426 ± 35.0	376 - 450	6
104	78 ± 10.1	71 - 92	18
105	127 ± 50.0	73 - 183	44
106	153 +-18.0	129 - 170	11

Table 16
Diurnal variation in serum KS (ng/ml), n=6, mean, standard deviation and range.

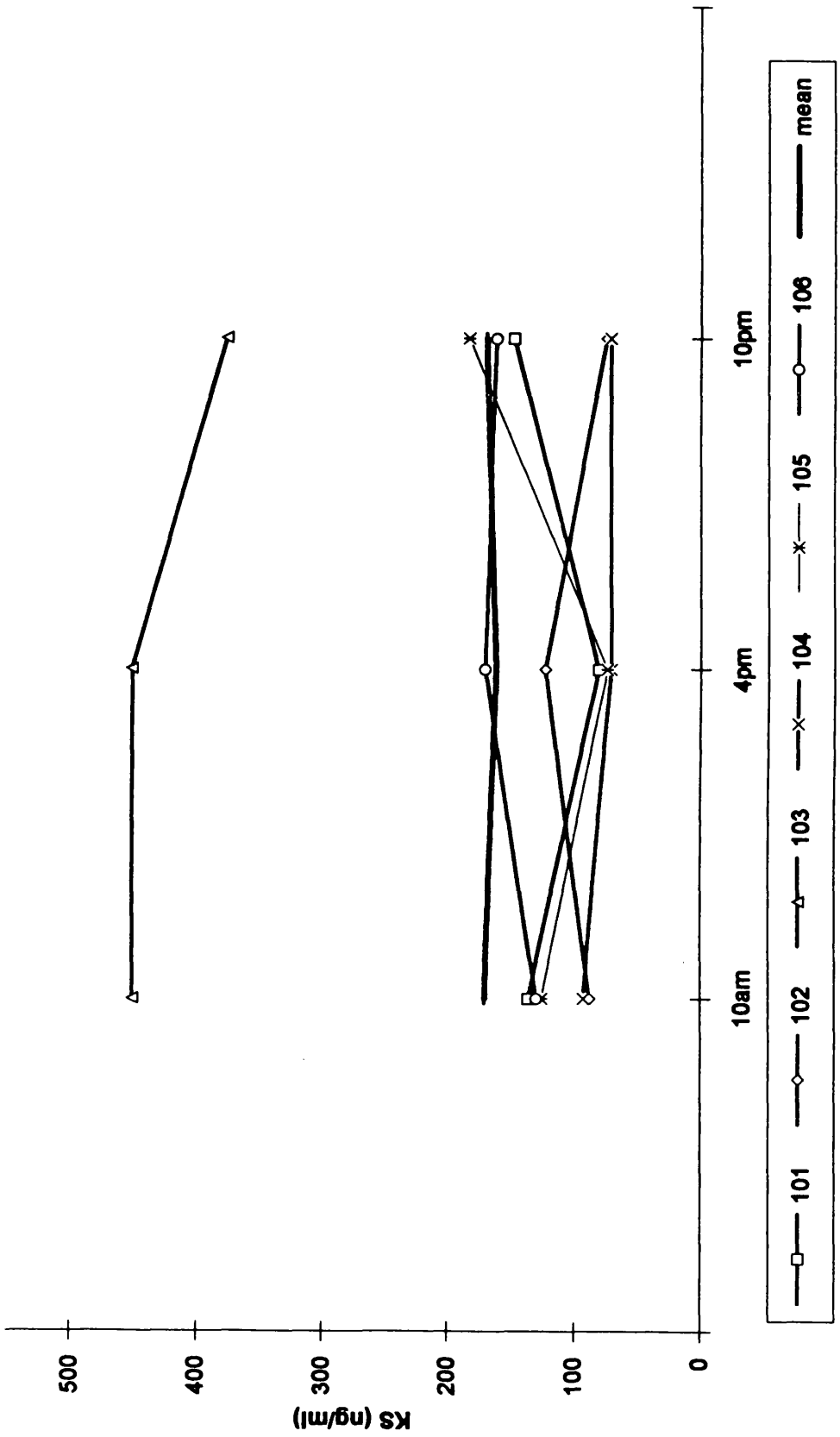

Figure 20
Diurnal variation in serum KS levels , n=6

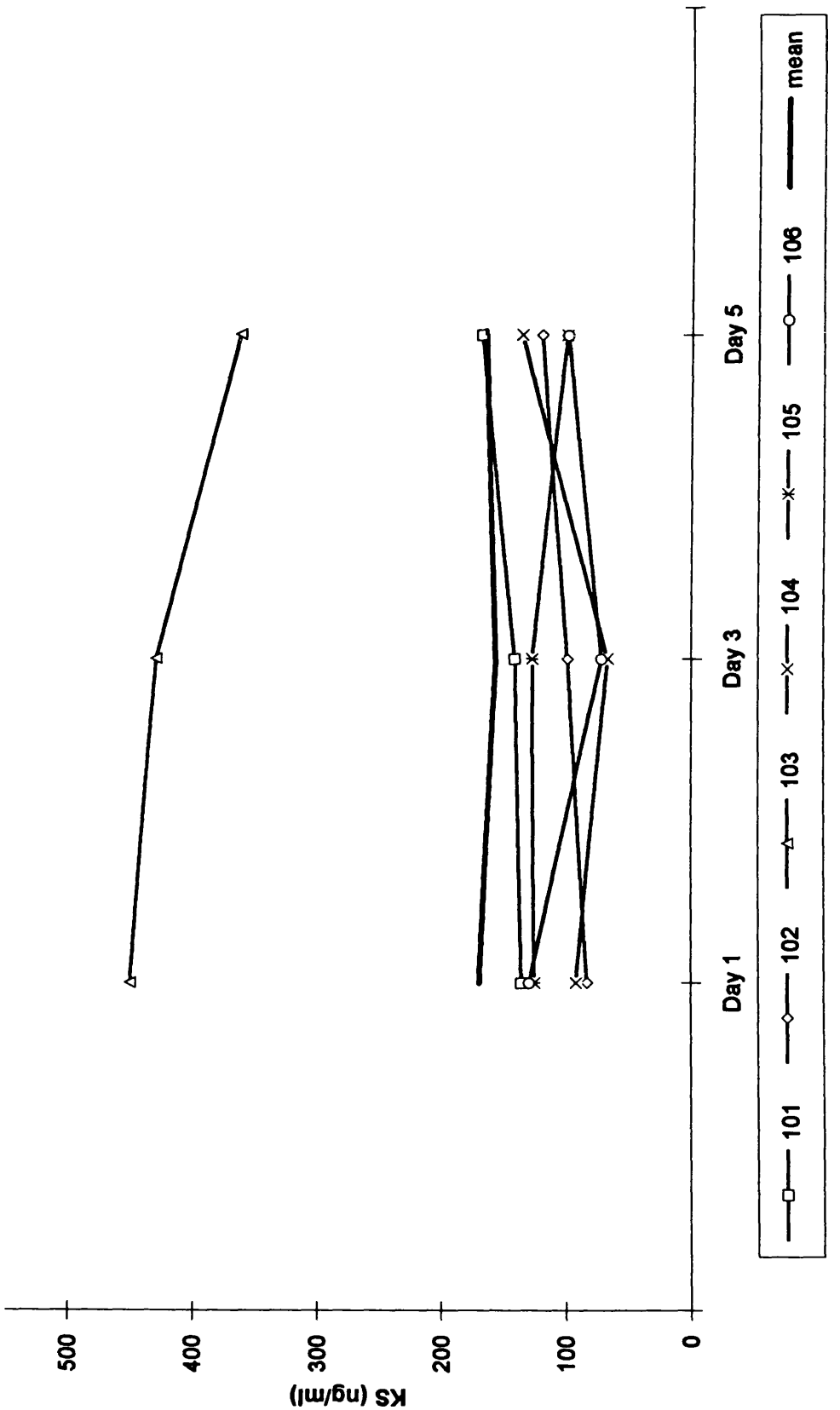

Figure 21
Day to Day variation in serum KS levels, n=6

5. EXPERIMENT 4A
STUDIES ON SERUM KS IN EXERCISING HORSES
OBJECTIVES

The purpose of this experiment was to determine the optimum time to blood sample normal horses post-exercise and evaluate whether serum KS levels were affected by a period of "acute" exercise.

5.1. Methods

An 8 years old Thoroughbred mare, used as a general riding horse / competition animal (eventer), exercised daily, was used as a control animal to determine if there was an optimum time to blood sample horses, following a period of acute exercise. This was carried out as a preliminary to taking samples from racehorses in training. The animal was selected on the basis of no history of joint disease or any form of orthopaedic injury. No joint abnormalities were detected on visual examination and palpation. There was no pain or lameness associated with flexion of the carpal, tarsal and metacarpophalangeal / metatarsophalangeal joints.

The animal was given a 45 min exercise session of trotting, cantering, jumping and galloping (moderate to high intensity exercise), rather than a standardised exercise test. All blood samples (10 ml/sample) were collected by jugular venepuncture into vacutainer tubes containing no anticoagulant. A blood sample was collected prior to the onset of exercise, and a second sample was collected immediately post-exercise. Further samples were collected at regular intervals up to 270 minutes post-exercise.

5.2. Results

Results are shown overleaf as ng/ml of KS equivalents. Results are plotted graphically in Figure 22. The first sample was taken at 10.30 am and subsequent samples were collected up to 4 pm. From the values obtained, the percentage changes calculated and the graphical evidence, it was concluded that a period of acute exercise appeared to cause an elevation in serum KS levels, (53 per cent change (increase) from baseline pre-exercise level) which took place during, or as an acute consequence of exercise in this animal. However, within 30 minutes of cessation of exercise, KS levels were starting to decrease and by 60 minutes post-exercise, values were only 9 per cent greater than the baseline value.

On the basis of these preliminary results, it was decided to bloodsample the group of racehorses in training immediately pre and post-exercise as well as one hour post-exercise to determine if acute exercise significantly affected serum KS levels in these animals.

TIME	KS (ng/ml)	% change from baseline
pre-exercise	141	100 (baseline)
5 min post	217	153
30 min post	159	113
60 min post	154	109
90 min post	153	109
150 min post	155	110
210 min post	97	70
270 min post	94	67

Table 17
Serial estimation of serum KS levels pre and post-exercise in one control animal.
All results are expressed as KS equivalents. The percentage change in serum KS from the pre-exercise value (baseline denoted here as 100 per cent) is shown.

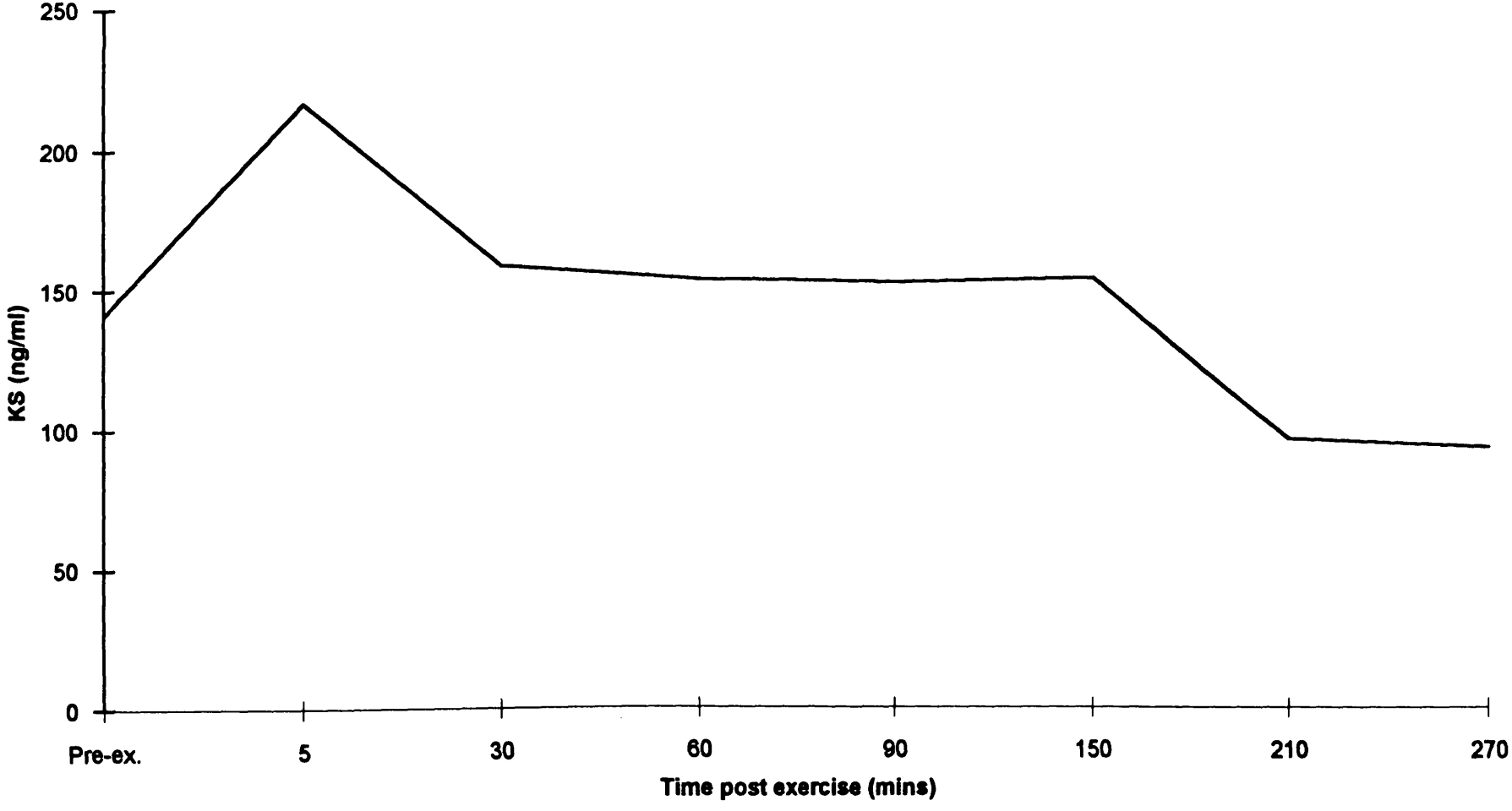

Figure 22
Serum KS levels pre and post-exercise in one control animal

6. EXPERIMENT 4B
INVESTIGATION OF THE EFFECT OF ACUTE EXERCISE ON SERUM KS LEVELS IN A GROUP OF RACEHORSES IN TRAINING

6.1. INTRODUCTION

A group of six racehorses in training were blood sampled before and after exercise to investigate whether there was any significant change in serum KS levels with a period of acute exercise. The age and sex of all animals was recorded (Appendix 14).

These horses were defined as normal within the context of the current study on the basis of no history of previous joint injury or other orthopaedic problems. On visual inspection and examination, there was no evidence of lameness or joint abnormalities, e.g. fluid distension or bone abnormalities. The animals were given a routine workout for that stable rather than a standardised exercise test. The exercise consisted of trotting, cantering and galloping for a period of 45 mins to 60 mins (moderate to high intensity exercise). All horses had been box rested the day prior to the test (trainer's day off).

The sampling time intervals for Test 1 were determined from the results of the one control animal exercised and serially sampled post-exercise. The racehorses were exercised and sampled on the same weekday as the control horse to eliminate any possible "day" effect. The pre-exercise sample was collected by jugular venepuncture from all animals in the same time period. A volume of 10 ml of blood was collected on each sample occasion. A second sample was collected within 5 mins of cessation of exercise and a third one hour later. As the 6 animals sampled were exercised in groups, the second and third samples were obtained over a four hour period, from 9.30 am to 1pm.

The experiment was repeated two weeks later (Test 2) on the same weekday, however, only four of the original six horses were available for sampling. It was decided, from analysis of the results *(see Results section)* obtained on the first exercise test, to sample these animals on two occasions, i.e. immediately pre-exercise, and five minutes post-exercise. The horses were given the same exercise routine as on the first test occasion. Samples were stored at 4 °C on collection and were processed as described earlier (Experiment 1) within 3 hours of collection.

6.2. Results

The values of KS obtained on the exercise tests are shown in Tables 18 and 19. The values obtained for each animal are plotted in Figure 23 and Figure 24. The mean values for the two groups at each sampling time in Tests 1 and 2 are also plotted. From Figure 23 and Figure 24, there was a trend for serum KS levels to increase with a period of acute exercise. It was decided to analyse the data non-parametrically as in this small group of horses the data appeared to be skewed on normality plots. Previous studies in horses have assumed the data is normally

distributed (Alwan and others 1990; Yovich and Carroll 1991), however this was not the case in the current study. A Wilcoxon-signed Rank test was used (p value < 0.05, 94% CI).

Analysing results, there was a statistically significant increase between pre and immediately post-exercise values, p=0.036, median —30, 94% CI [-71, -13.5]. There was also a statistically significant increase between values pre-exercise and those obtained one hour post-exercise, p=0.036, median —32, 94% CI [-73, -9]. There was no significant difference in values obtained immediately post-exercise and one hour post-exercise.

In Test 2, (n=4), there was an increase in serum KS levels as shown in Figure 24. This increase was however, not statistically significant.

Horse	Pre-exercise	5 mins post-exercise	1 hr post-exercise
No.1	69	90	77
No.2	58	140	136
No.3	65	71	75
No.4	52	73	74
No.5	70	130	138
No.6	53	92	95
MEAN	61	99	99

Table 18
Test 1. Serum KS levels in racehorses before and after a period of exercise (60 mins).
Results expressed as ng/ml of KS equivalents.

HORSE	Pre-exercise	1 hr post-exercise
No.3	78	137
No.4	74	74
No.5	72	78
No.6	68	100
MEAN	73	97

Table 19
Test 2. Serum KS levels in racehorses (n=4) before and after a period of exercise (60 mins).
Results expressed as ng/ml of KS equivalents.

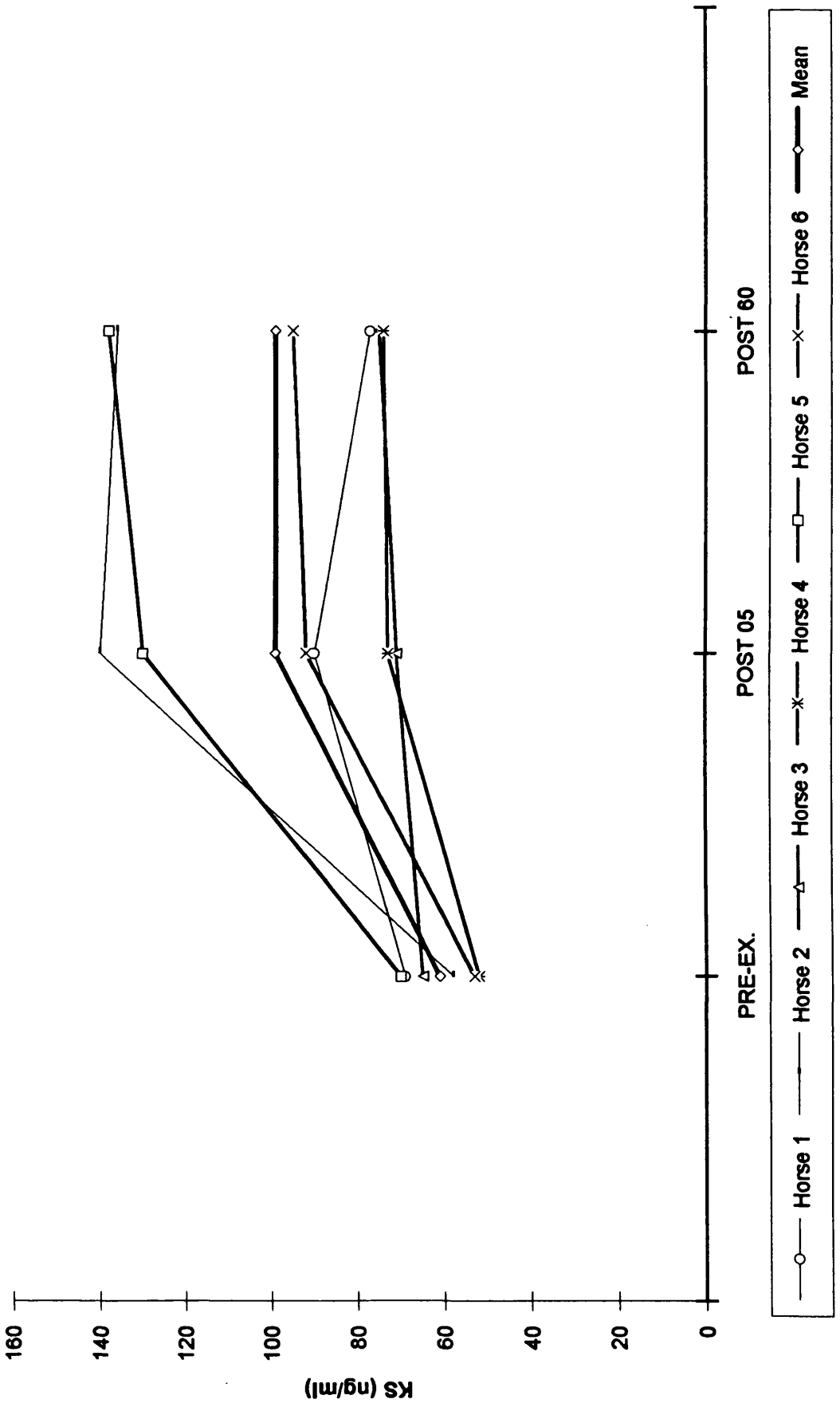

Figure 23

Test 1. Serum KS levels (ng/ml) pre and post exercise (5 mins and 60 mins) in a group of racehorses, n=6.

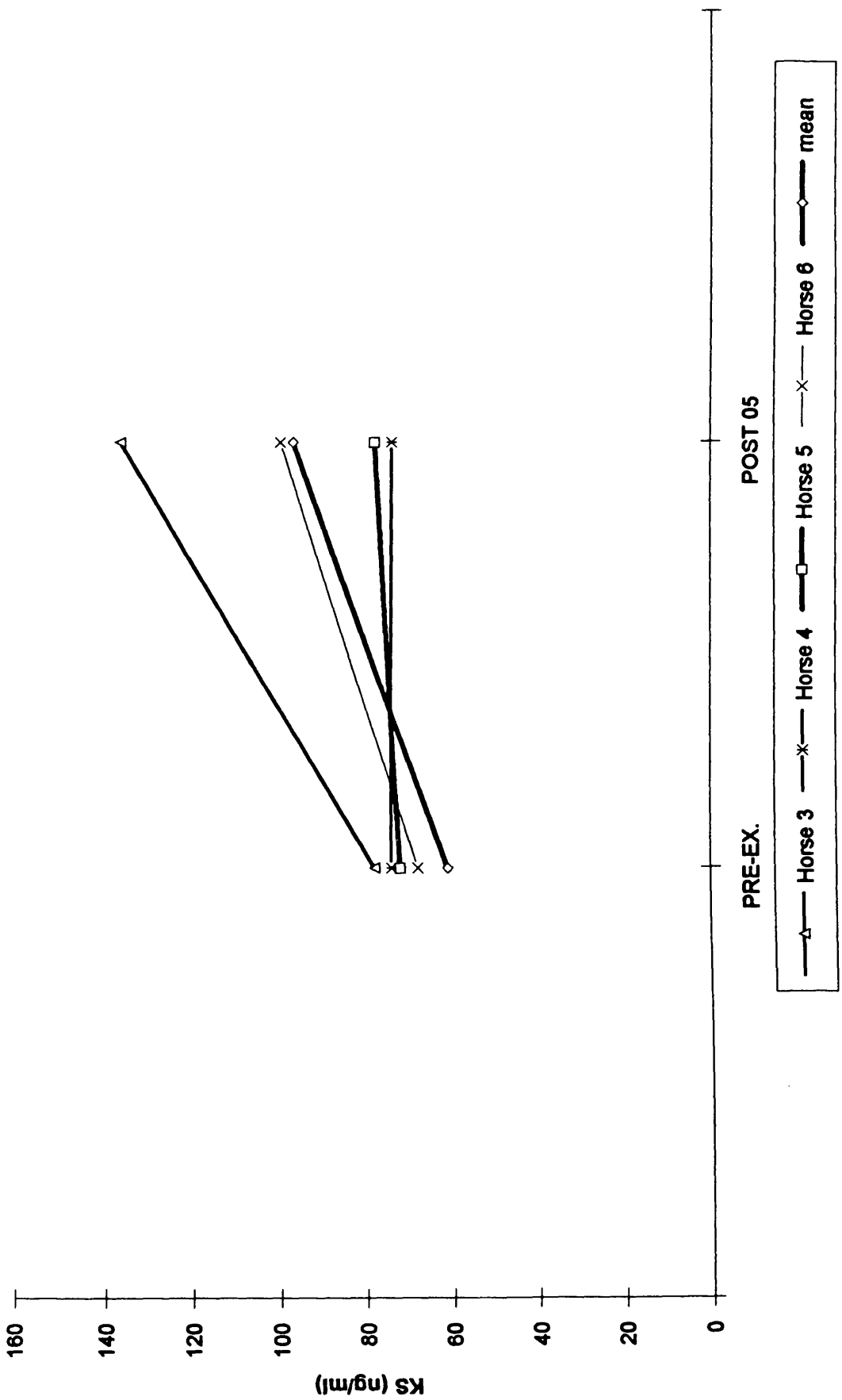

Figure 24

Test 2. Serum KS levels (ng/ml) pre and post exercise (5 mins) in a group of racehorses, n=4

CHAPTER IV

MEASUREMENT OF SERUM KS IN HORSES UNDERGOING SURGICAL TREATMENT FOR OSTEOARTHRITIS

1. OBJECTIVES

The objective in measuring KS in the three cases presented here was to monitor the effects of surgery on post-operative serum levels of KS and the response of the cartilage in the post-operative period. One animal was monitored for a period of fifteen weeks post-operatively, while the other two could only be sampled during their stay at Glasgow University Veterinary School (GUVS). Clinical history and findings are detailed as well as the measurements of KS.

2. SUBJECTS

Case 1.

A five year old Thoroughbred flat racehorse was presented with a history of lameness and distension of the palmar and dorsal pouches of the left metacarpophalangeal joint one month previously following a race.

2.1. Examination

At examination, there was moderate distension of the palmar pouch of the joint with swelling and heat localised on palpation over the medial sesamoid bone. Direct palpation over the medial sesamoid and distal part of the medial branch of the suspensory ligament also elicited a pain response.

At walk, the animal did not appear lame, but at trot, there was a Grade 1 out of 5 lameness evident (Stashak 1987). There was an obvious pain response on flexion of the joint and immediately following this flexion test, the horse was 1 ½ out of 5, to 2 out of 5 lame at trot.

2.2. Diagnostic procedures

A four point nerve block (palmar nerves anaesthetised just proximal to the palmar pouch of the metacarpophalangeal joint and palmar metacarpal nerves just over the button of the splint bone), did not improve the lameness, however, the animal was less painful on flexion of the joint and there was no increase in lameness following the flexion test.

Intra-articular anaesthesia of the affected joint was carried out, using 5 ml of mepivicaine (Intraepicaine, Arnolds Veterinary Products Ltd. U.K.) injected aseptically into the joint. Prior to injection of the local anaesthetic agent, a synovial fluid sample was collected (2 ml) and placed in EDTA for cytological evaluation and in a tube containing no anticoagulant for KS estimation. Cytology results are shown in Table 20. The synovial fluid sample appeared grossly normal. A sample was also taken for serum KS estimation. The horse was evaluated at walk and trot and the lameness was noted to have improved approximately 50 per cent. Ultrasonography of palmar metacarpal soft tissue structures was carried out and there was evidence of gross disruption of the linear

ligament which was enlarged to twice its normal size. There was evidence of insertion sesamoiditis which was confirmed on radiography.

Standard views of the metacarpophalangeal joint were taken, as well as a flexed lateral view (Figure 25). Radiography revealed a severe advanced medial sesamoiditis with evidence of bone remodelling. A small, barely radiodense opacity was noted in the palmar pouch just at the point where articular cartilage stops and the soft tissues of the joint start on the palmar aspect of the third metacarpal bone. On close inspection of the apical part of the medial sesamoid bone, an old fracture bed was visible with evidence of one small chip in place.

2.3. Surgical treatment

Arthroscopy was carried out under general anaesthesia. Prior to distension of the joint, a synovial fluid sample was taken for KS evaluation. The arthroscope was placed in the medial palmar pouch of the joint. There was evidence of inflammation of the synovial membrane and palmar aspect of the third metacarpal bone. Debridement of the hypertrophied soft tissue in this area resulted in the removal of the small chip of bone seen on radiography. Examination of the medial sesamoid bone revealed extensive damage to the abaxial surface with loss of bone and disruption of the insertions of the suspensory ligament. Two small apical chip fractures were also removed. Curettage was carried out until healthy bone was identified and the joint was extensively lavaged. Further examination of the joint and palmar pouch did not reveal any other abnormalities. The horse was given 4.4 mg/kg body weight of phenylbutazone (PBZ), (Phenyzene, C-Vet Ltd. U.K.) intravenously prior to recovery from general anaesthesia. A Robert Jones bandage was placed on the affected limb and the animal confined to box rest in the post-operative period. On the first post-operative day, the horse was given 1 mg/kg body weight of PBZ (Equipalazone, Arnolds Vet. Products, U.K) orally twice daily, followed by 0.5 mg/kg/body weight of PBZ orally twice daily for two further days. Post-operative recovery advice consisted of bandaging the affected joint for 6 weeks. Strict box rest was recommended for 14 days post-operatively, followed by in-hand walking for 5 to 10 mins twice daily, increasing to 20 to 30 mins twice daily. Approximately 6 weeks after surgery, the horse was turned out to grass.

Serum samples (10 ml) were taken for KS evaluation at 2 and 5 days post-operatively, while the animal was at GUVS, and then 7 days later, when the horse had returned to the training establishment. Serial samples were collected at 14 day intervals on two further occasions until the animal was turned out to grass for an eight week period. It was not possible to collect samples for serum KS estimation from the horse during this time. A final sample was collected following the animal's return to training.

At the time of collection of the last sample, the horse had begun roadwork, consisting of daily walking and trotting, with a view to starting a career as a hurdler/chaser. The values of KS measured from the affected joint, and serum KS levels are shown in Table 21, together with the post-operative recovery programme. The data is shown graphically in Figure 26.

RBC (x10 ^{12}/L)	0.02
Hb (g/dL)	0.0
WBC (x10 ^{9}/L)	1.9
Neutrophils	0.08
Lymphocytes	0.46
Monocytes	0.00
Basophils	0.00
Eosinophils	0.00
Macrophages	1.37

Table 20
Synovial fluid cytology results for Case 1.

Day of sample	**KS equivalents (ng/ml)**	**Peri/post-operative programme**	**Per cent change from baseline**
Day -3	74 2518(synovial)	pre-operative	68 %
Day 0 (Baseline)	108.24 3887(synovial)	intra-operative	100 %
Day 2	97	Box rest	89 %
Day 5	81	Box rest	74 %
Day 12	87	Box rest	80 %
Day 14	***	Start walk-out, 5 min. BID	***
Day 26	102	Walking out in hand, 10 min. BID	94 %
Day 41	86	Walking out, 20 min. per day	80 %
Day 42	***	Out to grass for 8 weeks	***
Day 106	40	Back in work one week	36 %

Table 21
Pre and post-operative serum KS concentration for Case 1.
Key: *=not blood sampled. Baseline (100 per cent) is the value obtained on the day of surgery.**

Figure 25. Flexed lateromedial view of the left metacarpophalangeal joint in Case 1. There is insertion sesamoiditis and a radiodense opacity in the palmar pouch of the joint just at the junction of the articular cartilage and soft tissues on the palmar aspect of the third metacarpal bone.

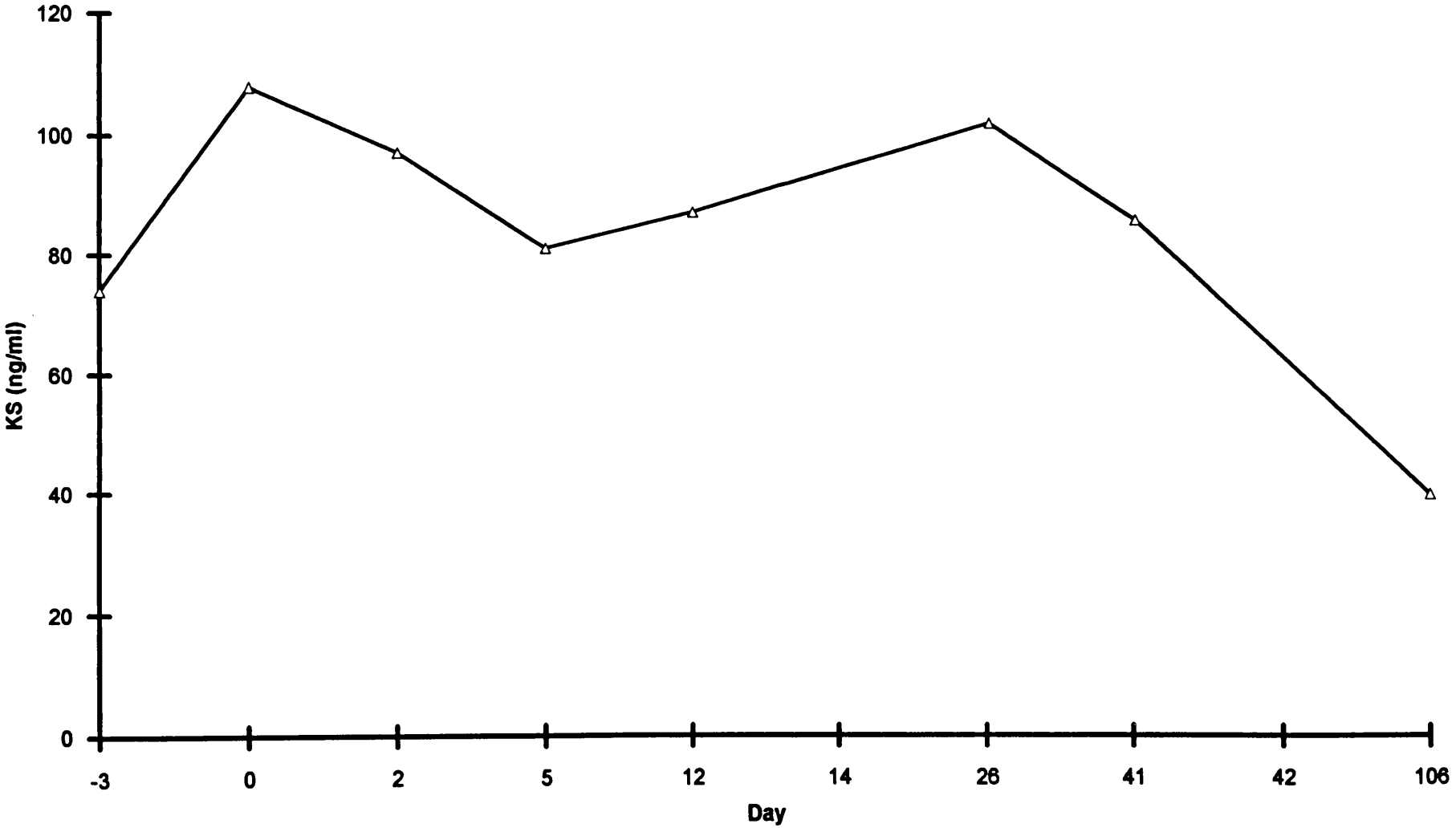

Figure 26. Serum KS levels (ng/ml) in Case 1 pre-operatively and serially post-operatively

2.4. Subject : Case 2

A 6 years old Thoroughbred mare, used for general riding purposes, was referred with a history of intermittent lameness of approximately 6 to 9 months duration. A tentative explanation for the onset of lameness was the occurrence of a fall while the animal was at grass.

2.5. Examination

At examination, there was evidence of mild left gluteal muscle atrophy. A pain response was elicited on flexion of the left metatarsophalangeal joint. There was no evidence of joint distension and no other abnormalities were detected in the limb. At walk, the animal was 1 out of 5 lame and this increased to 2 out of 5 lame at trot. Following a flexion test on the left metatarsophalangeal joint, the lameness increased in severity.

2.6. Diagnostic Procedures

Standard radiographic views were taken of the affected joint. There was a medial plantar process fracture of the first phalanx (P1) visible on radiography (Figure 27). This was associated with a lytic area surrounded by a sclerotic rim in the plantar process of P1. A diagnosis of DJD was confirmed on radiography. The right metatarsophalangeal joint was normal in appearance on radiography.

2.7. Surgical Treatment

Arthroscopy was carried out under general anaesthesia. Prior to joint distension, a 2 ml aliquot of synovial fluid was collected for KS estimation (tube containing no anticoagulant) and for routine synovial fluid cytology (EDTA). Cytology results are shown in Table 22. A blood sample (10 ml) was also collected for serum KS evaluation.

On arthroscopy, there was evidence of synovial proliferation within the joint and villous changes indicative of a chronic synovitis. The bone fragment was located in the distal portion (medial) of the plantar pouch and it was totally enclosed within the plantar synovial membrane. The fragment was removed by sharp dissection and the defect in the plantar process of P1 was curetted down to fresh bleeding subchondral bone. Peterson and Ryden (1982) reported that Type 1 osteochondral fragments located on the palmar aspect of the metacarpophalangeal joint could result in degenerative joint disease. The bone fragment was submitted for histopathology. The fragment was found to be composed mainly of dense, mature, poorly cellular but slightly disorganised bone tissue surrounded by dense collagen, containing occasional knots of bloodvessels. There was no evidence of an epithelial or synovial lining. There was no evidence of an inflammatory infiltrate. The joint was thoroughly lavaged, intravenous PBZ administered (as in Case 1) and routine post-operative care was given. The animal was confined to box rest and placed on a 3 day course of oral PBZ (1mg/kg body wt.) twice daily. Serial samples were taken for serum KS estimation at 2 and 4 days post-operatively until the animal was taken home and

was no longer available for bloodsampling. The synovial fluid KS and serum KS results are shown in Table 23.
Post-operatively, the horse was box-rested for a further 7 days following return to its owner. Subsequently, in-hand walking exercise for 5 mins twice daily was begun. At 4 weeks post-operatively, the period of exercise was increased up to 15 mins twice daily. The horse was then turned out to grass, and was placed back in a graduated exercise programme, not returning to full work for at least 12 weeks.

RBCx 10^{12}/Litre	0.05
Hb g/dl.	0.0
WBCx10^{9}/Litre	0.2
Neutrophils	0.0
Lymphocytes	0.11
Monocytes	0.00
Eosinophils	0.00
Basophils	0.00
Macrophages	0.09

Table 22
Synovial fluid cytology results for Case 2.

Day	Ks equivalents (ng/ml)	Peri/post op. programme	% change from baseline
Day 0	96 9,238(synovial)	Intra-operative sample	100% (baseline)
Day 2	175.7	Box rest	183 %
Day 4	124.8	Box rest	130 %

Table 23
Serum KS concentration (intra and post-operative) for Case 2.
The synovial fluid KS result is shown in brackets. Day 0 = the day of surgery and baseline (100 per cent).

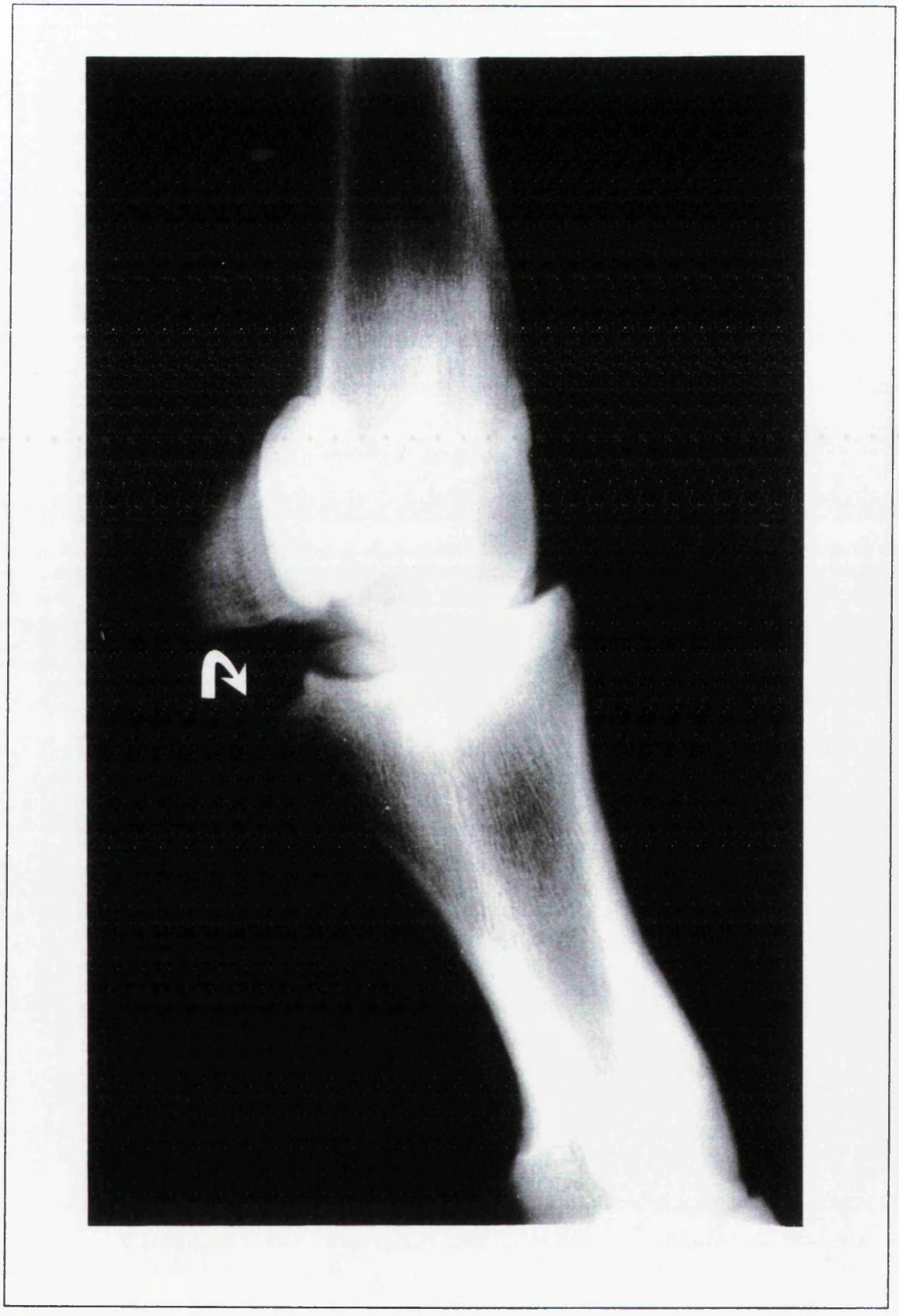

Figure 27. Dorso-medial palmarolateral 45 ° oblique view of the left metatarsophalangeal joint for Case 2. There is a medial plantar process fracture on P1, associated with a lytic area surrounded by a sclerotic rim in the plantar process of P1.

2.8. Subject: Case 3

A five year old Thoroughbred gelding, used as a Flat racehorse, was presented with acute onset, severe lameness which had started during training. The lameness was attributed to the right forelimb.

2.9. Examination

At presentation, the palmar pouch of the right metacarpophalangeal joint was distended. There was pain on flexion of the joint and increased lameness following a flexion test (2 out of 5 lame at trot prior to flexion of the affected joint increasing to 3 out of 5 lame at trot after flexion).

2.10. Diagnostic Procedures

Standard radiographic views were taken and there was evidence of some remodelling of the dorsomedial aspect of the first phalangeal bone (P1), on the dorsomedial articular rim of P1 (Figure 28).

Intra-articular anaesthesia of the joint was carried out using 5 ml of mepivicaine (Intraepicaine, Arnolds Veterinary Products Ltd. U.K.). On examination at 20 min post-injection of local anaesthetic, there was decreased pain on flexion and an improvement in the lameness to 1 out of 5 lame.

2.11. Surgical Treatment

A right dorsal metacarpophalangeal arthroscopy was performed. Prior to joint distension, a synovial fluid sample (2 ml) was taken and retained for KS evaluation. Arthroscopy revealed extensive remodelling of the dorsal aspect of P1 with evidence, particularly to the medial aspect of the sagittal ridge, of cartilage damage. Opposite this area was a deep score mark on the medial aspect of the distal third metacarpal bone articulation with the metacarpophalangeal joint. There were linear "wear" lines on the medial aspect of the third metacarpal bone and evidence of enlargement of the dorsal capsule fibrous mass. There was also obvious villous hypertrophy. The cartilage lesions on the medial side of the dorsal aspect of P1 were removed and the joint extensively lavaged. Intravenous PBZ was administered (as in Case 1 and 2). Routine post-operative care was administered (as already described) and the horse placed on a 5 day course of oral PBZ (1mg/kg body wt. twice daily). Samples were taken for serum KS evaluation at two and four days post-operatively (Table 24).

Day	KS equivalents	Peri/post-op. programme	% change from baseline
Day 0	75.9 (3,272-synovial)	Intra-op.	100% (baseline)
Day 2	169.3	Box rest	222.9%
Day 4	114	Box rest	154%

Table 24

Serum KS values (intra and post-operative) for Case 3.

Intra-operative synovial fluid KS concentration is shown in brackets. **Day 0** : day of surgery.

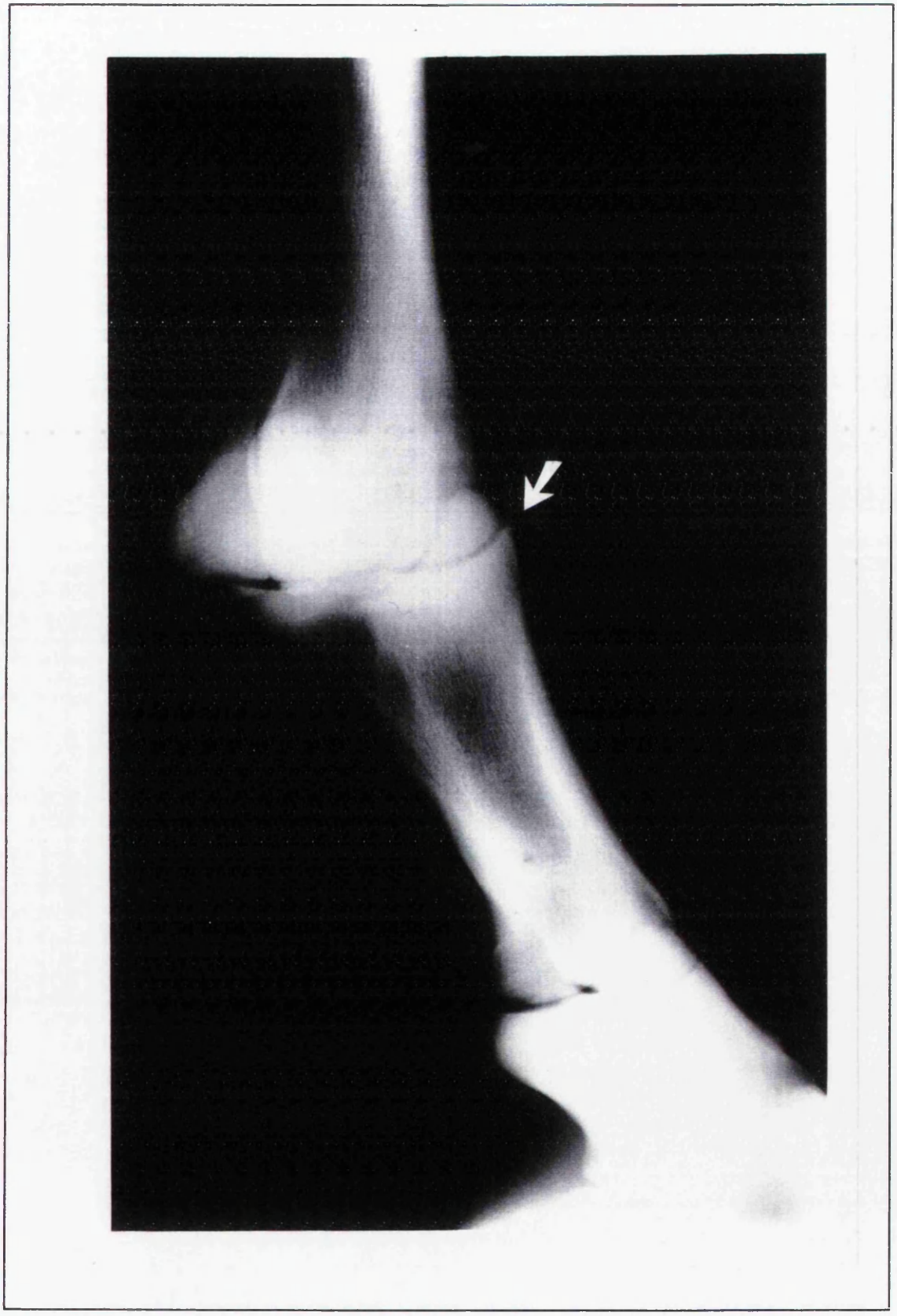

Figure 28. Dorso-lateral palmaromedial 45 ° oblique view of the right metacarpophalangeal joint in Case 3. There is remodelling of bone on the dorsomedial aspect of the first phalanx, on the dorsomedial articular rim of the first phalanx.

2.12. Results

In the three cases investigated, it was found that there was a significant increase in serum KS measured two days post-operatively in Cases 2 and 3, where serum KS had shown a change from baseline in Case 2 (85 per cent increase)and 123 per cent increase over baseline with Case 3. However, by Day 4, the levels of serum KS in both horses had changed to 30 per cent greater than baseline (Case 2) and 54 per cent greater than baseline (Case 3). With Case 1, the levels of KS measured in synovial fluid taken just before surgical intervention were 54 per cent greater than those measured 3 days prior to surgery and serum levels just before surgical intervention were 32 per cent greater than those taken pre-surgery. There was a decrease in serum KS levels from baseline at 2 and 4 days post-operatively. Over the ensuing sampling period post-operatively, serum KS values remained within ± 10 per cent of the baseline value. There was no marked increase in serum KS values when the horse returned to walking exercise two weeks post-operatively. Synovial fluid cytology in the two horses sampled was within normal limits (Todhunter and Lust 1992). Serum KS values from the three horses were within the range of values observed in the group of racehorses in Experiment 4.

CHAPTER V
GENERAL DISCUSSION

1. THE ELISA ASSAY

1.1. Factors influencing the quantitation of KS in biological samples using the ELISA assay.

The development of highly specific immunoassays has provided a sensitive method for *in vivo* quantitation of KS in articular cartilage and serum (Funderburgh and others 1983; Thonar and others 1985; Ratcliffe and others 1988). These analyses are performed using a variety of different monoclonal antibodies specific for KS epitopes and quantitation of KS is achieved by comparison with cartilage PG standards from a variety of different animals and tissues. The ELISA in the current study uses a well characterised MAb (1/20/5D4) which has been shown to be highly specific for skeletal and corneal KS (Caterson and others 1983).

1.2. Characteristics of the KS epitope

The epitope region recognised by the MAb (1/20/5D4) consists of segments that contain at least three repeating units of sulphated galactose-sulphated acetlyglucosamine (Mehmet, Scudder, Tang, Hounsell, Caterson and Feizi 1986). These highly sulphated sequences have been determined to be located on the distal, non-reducing end of KS chains (Stuhlsatz, Keller, Becker, Oeben, Lennartz, Fischer and Greiling 1989). The epitope detected by this MAb is present only in the longest KS chains that represent less than 15 per cent of the total KS chains present in bovine nasal cartilage aggregating PGs. It has been suggested that a decrease in the size of the KS bearing molecule was accompanied by a decrease in the amount of KS epitope detected using a variety of MAb. Thonar and others (1991b), state that this does not appear to be a problem in the assay used in the current study, i.e. differences in the size of the molecules on which the epitopes are presented do not translate into differences in the amount of epitope detected. Evidence to support this is also found in the study of Thonar and others (1985), in which highly purified preparations of KS from human costal or bovine corneal tissue yield inhibition curves in an ELISA that are identical to those obtained using undigested PGs from these tissues.

It is assumed that the content of the KS epitopes and their presentation in the standard and unknown samples are similar. However, KS isolated from different animals and different tissues or at various developmental stages shows considerable variation in their biochemical analyses and their biophysical characterisation (Caterson and others 1989).

Several factors contribute to the structural heterogeneity of KS in biological samples and thereby influence the presentation of epitope for immunoassay. These include the degree of sulphation and the variable distribution of regions of the non, mono and disulphated disaccharide units within each individual KS chain (Oeben, Keller, Stuhlsatz and Greiling 1987). Variation in chain length

with the possibility of multiple epitopes per chain (polyvalency) especially in the longer KS chains and the close attachment of a number of KS chains on a single polypeptide that may result in co-operative binding, will influence the results obtained (Caterson and others 1989). In the current study, the results of the spike assay were in agreement with the observations of Caterson and others (1989), demonstrating that the bivalent nature of the immunoglobulin molecule and the occurrence of multiple epitopes within the KS chain can affect the apparent quantitation of KS, leading to an overestimation of the quantity of epitope present. Their study also concluded that there may be differences in evaluating KS from normal and pathological specimens as the KS isolated from diseased samples may differ in both their chain size and in the relative abundance of a given epitope within the GAG chain.

When attempting to quantify KS, it must be borne in mind that it is only a proportion of the total KS that is actually being measured, i.e. the antigenic epitope recognised by the monoclonal being used in the assay (in this study, 1/20/5D4). The amount of KS in a sample is referred to as nanogram equivalents of IRS, or WS (as used in the current study and referenced against IRS). Thonar (1989b), states that different anti-KS antibodies or different immunoassays using the same or different antibodies, can yield varying results. In the ELISA used in this study, he observed that when samples were pre-treated with papain, they lost no more than 35 per cent of their antigenicity. In contrast, when another monoclonal antibody, MZ15 was used in the same assay this loss of antigenicity became greater than 95 per cent. This must be borne in mind when comparing the results of different studies.

1.3. Volume turnover of synovial fluid in relation to quantifying KS

Several factors govern the intra-articular concentration of macromolecules such as KS and other GAGs. These include macromolecule input rate, the flow of water across the synovial lining and the properties of the synovial intercellular matrix (Levick 1992). Macromolecule concentration is inversely related to the volume turnover rate, as the input macromolecule is constantly being diluted in the stream of synovial fluid passing through the joint cavity. This is especially important in arthritis where fluid turnover rates can be increased by 100 per cent to 300 per cent and where concentration values taken in isolation can be very misleading (Levick 1992). The time-averaged volume turnover is estimated to be 2 to 4 $\mu l/hr/cm^2$ in normal human and rabbit knees (Levick 1987). In human arthritis the average volume turnover rate increases significantly (Knox, Levick and Mc Donald 1988), and this needs to be considered when interpreting intra-articular concentration data from affected joints. In both human and veterinary literature, KS is quantified in terms of ng per ml of KS equivalents rather than ng/ml of synovial fluid volume. The half-life of hyaluronan has been determined experimentally in horses (Levick 1992) in terms of volume flow and the effect of hyaluronan on the efflux of other macromolecules. Studies on KS in horses to date, however have not addressed the issue of the effect of synovial fluid volume (e.g. dilution effects in inflammatory arthritides/synovial effusion) or composition on results obtained. Efforts to quantify KS related to synovial fluid

volume have concentrated on referencing KS to total SGAG (Alwan and others (1990) and Thonar and others (1991b), however, this does not imply that they are measuring identical pathological processes within the joint (Carroll 1989). Another possible means of quantifying KS measurements in relation to synovial fluid volume would be to use urea and albumin dilution techniques, which have previously been used in the horse to quantify bronchoalveolar lavage fluid volumes (Mc Gorum, Dixon, Halliwell and Irving 1993).

1.4. Non-specific binding

It was thought that the highly negatively charged sulphated polyanion on KS would electrostatically interact with other biopolymers, however, the assay used in the current study has been shown to have no interference from electrostatic interactions and non-specific binding (Caterson and others 1989). Scott (1989) voiced the concern that in acute fulminating inflammatory conditions with gross cell destruction, liberation of usually highly cationic cell proteins could result in electrostatic interactions with the highly anionic KS epitope. However, Caterson and others (1989), have shown experimentally that the specific interaction of the monoclonal antibody with KS is very strong and that the possibility of non-specific binding in this assay is minimal.

1.5. Matrix effect and interactions with factors in serum

It has been stated that possible interaction of unknown factors in serum with the MAb may result in masking of the epitope. Baker (1989) found that KS may ionically interact with high density lipoprotein in a potassium bromide gradient, suggesting that this interaction was not salt-concentration dependent and that other unknown factors were involved. The mechanism by which serum disturbs the response is unknown (Price and Newman 1991). It may affect the antigen-antibody response as suggested above, or the separation procedure. In the current study, there was strong evidence of non-parallelism between the standard curve and equine serum samples, which, (although it did not significantly affect the actual concentrations of KS measured) suggested that there may have been a matrix effect influencing the recognition and measurement of KS in equine serum.

1.6. Metabolism of KS

Proteoglycans, on leaving the synovial fluid compartment via the subsynovial lymphatics, may be further degraded before entering the blood, from which they are eliminated via the liver and kidneys (Thonar and others 1985). Exposure of terminal galactose residues on the KS chains and oligosaccharides results in a marked increase in the rate of elimination of fragments of all sizes, suggesting that binding of fragments to galactose receptors on hepatocytes represents an important pathway of elimination from blood (Maldonado and others 1989). KS fragments are also excreted in urine (Thonar and others 1989b). It may be advisable to evaluate liver function in order to accurately interpret serum KS levels, however, Thonar and others (1989b) state that serum KS levels are not affected to the same degree as hyaluronan, in the presence of liver disease.

Tests of liver function were not carried out in all animals used in the current study. Studies in human and veterinary literature have not addressed the potential effects of liver pathology on the metabolism of KS.

1.7. Discussion on the various methods used to assay KS in equine studies

Alwan and others (1990) used an ELISA to measure KS similar to that used in the current study, except that microtitre plates were coated with equine articular cartilage PG monomer and a pure equine articular cartilage standard was included in each plate. The standard inhibition curve was constructed from values obtained by addition of horse KS standard to the coated plate in increasing concentrations. The monoclonal anti-KS antibody used was the same as in the current study.

Todhunter and others (1993) used an ELISA method which was essentially that of Thonar and others (1985), with minor modifications by Leipold and Lust (1989). They used pooled equine plasma as the calibrated control in the ELISA of plasma, and pooled equine synovial fluid as the control in the ELISA of synovial fluid samples. Their rationale for using these calibrated controls was that a significant number of the antigenic KS chains contain more than one epitope (Thonar and others 1991b), and KS chains in synovial fluid may contain different numbers of epitopes per molecule, compared with those in plasma. The slope of the control curve for the concentration of KS in plasma when plotted against optical density was less than when that of the synovial fluid KS was measured. An indirect method of establishing the concentration of KS in the controls was used as they suggested that the purified skeletal KS standard does not react strongly with the anti-KS monoclonal antibody in the ELISA. A possible explanation offered for this is that the conformation of the sulphate-oligosaccharide moieties on the KS, which are recognised by the antibody, have been altered during purification. This method involving the use of calibrated controls and indirectly establishing the concentration of KS in the standards has not been described elsewhere in the literature. The ELISA used in the current study, using WS referenced against IRS has been well described in human literature and widely used. In the current study, it was found to be reproducible from day to day, specific and reasonably sensitive, given the occurrence of polyvalency of the KS epitope (Caterson and others 1989), which is inherent to the KS molecule and is likely to be encountered using other assay methods.

1.8. Validation of the KS assay used in the current study

The inter-assay C.V. in the current study ranged from 10.5 per cent to 11.5 per cent. This corresponded well to that of Thonar (1993, unpublished data) which was 11.0 per cent. The fact that the interassay C.V. remained relatively consistent over the three batches of plates made, suggested that the uniformity of plate coating did not differ greatly from batch to batch. The intra-assay C.V. of 4.3 per cent was also acceptable (Thonar 1993 unpublished data).

Thonar and others (1991), report that repeated freezing and thawing and long-term storage (up to 4 years) at —50 °C did not cause any loss of antigenicity of

serum KS. In an earlier study, (Thonar and others 1985), it was reported that repeated freezing and thawing (storage temperature not specified), of serum or plasma did not in any way affect the level of serum KS measured (SD <2 per cent). In the current study, storage of serum at —20 °C for a period of 8 weeks resulted in a measurement that was 37 per cent less than the original measurement. Taking into account the allowed interassay C.V. of 10.9 per cent this measurement indicated deterioration in the sample over time. The sample stored at 4 °C gave a measurement 41 per cent less than that of the original sample. The sample stored at room temperature could not be assayed due to fungal contamination. From these results it would appear that it is preferable to store samples at a temperature of at least —50 °C to prevent deterioration in KS antigenicity.

In the spike assay, the addition of exogenous KS (WS) to equine serum and the increase recorded correlated with the findings of Seibel and others (1989), which showed that there were no blocking factors in serum from normal human patients. On both occasions on addition of the two spike samples of exogenous KS (WS) to equine serum samples, a reading greater than that expected occurred. The greater than predicted KS concentration may be explained by the fact that several domains of the KS molecule containing the epitopes recognised by the monoclonal antibody can occur in individual KS chains (Oeben and others 1987). Therefore there is a potential for the bivalent immunoglobulin molecule to bind more than one epitope in a given KS chain (Caterson and others 1989), leading to an overestimation of the amount of KS actually present. The magnitude of this overestimation has not been quantified to date and it is accepted that this is one limitation of the ELISA method used to measure KS.

2. STUDIES IN NORMAL HORSES

2.1. Experiment 1

Previous studies in horses have defined "normal" animals as those showing no evidence of lameness on clinical examination and no history of lameness (Alwan and others 1990). These authors also used synovial fluid samples and in some cases, radiography, to confirm that their horses had no joint abnormalities.

Yovich and others (1991) defined their normal horses as those that showed no evidence of lameness at trot, no synovial effusion from the midcarpal joint (the joint studied in lame animals) and no evidence of radiographic abnormalities on lateral, oblique, dorso-palmar and flexed lateral radiographic views of the carpus. Synovial fluid was also analysed from the midcarpal joint of normal horses for routine cytology and total protein, however, results were not shown and no correlation with KS results was carried out. In the study of Little and others (1990) normal joints were classified as those in which there was no sign of synovial effusion or pain during life, no radiographic abnormalities and grossly normal cartilage on post-mortem examination.

In the current study, normal horses were defined as those which showed no evidence of lameness on clinical examination and no evidence of OA on gross inspection of the articular surfaces at post-mortem inspection. Although synovial fluid collected from each joint was visually inspected, cytological examination was not carried out. Alwan and others (1990) found that the mean number of synovial fluid white blood cells did not significantly differ between normal horses and those with OA, therefore, synovial fluid analysis was not routinely carried out in the horses used in this study for this reason and also for cost factors.

2.2. Definition of a "normal" joint

Poole and Meagher (1990), describe structural alterations known to occur in "normal" equine joints. Small defects that appear as dimples or linear infoldings in the articular surface occur as a result of occlusion of blood vessels located within the cartilage canals of the articular cartilage. These lesions develop as a result of focal death and collapse of cartilage tissue in the radial zone of the cartilage during endochondral ossification. These defects arise during skeletal growth, are apparently unassociated with the develpoment of osteochondrosis and do not predispose to the development of OA. The pattern of scoring or "wear" lines (Rooney 1969) occurs commonly in the metacarpophalangeal joint and metatarsopohalangeal joint, the radiocarpal and midcarpal joints and the tibiotarsal joints. They are found in mature horses of all ages and the mild form of the lesion, which is not necessarily progressive, is commonly observed at post-mortem examination. However, at some stage, they can become symptomatic, resulting in OA. They are believed to be self-limiting, degenerative changes produced as a result of the effects of synovial fluid turbulence following habitual lines of movement within the joint (Rooney 1969). These changes within the joint are accepted as normal features and would not be visible on radiography.

2.3. Findings in normal horse joints

In Experiment 1, Horse No. 3 had one small dimpled lesion (4 mm diameter) on the medial articular surface of the right distal radius with a corresponding lesion of a similar size on the articular surface of the radial carpal bone. Horse No. 6 had a dimpled area (5mm) on the articular surface of the ulnar carpal bone, seen when the right radiocarpal joint was opened. In these two animals, the KS levels recorded for these joints were not greater than those from the contralateral joint (Horse 3) and in Horse 6, levels from the right radiocarpal joint were very similar to those from the left radiocarpal joint. The contribution of these wear and tear lesions to the levels of KS released from or turned over in the joint is unknown. Although the clinical features of normal horse joints are reasonably uniform, there is variation in the articular cartilage within the range of "normal" that may influence the KS measurements obtained from these joints. However, in the current study, it did not appear to make a difference to the results obtained.

In the group of eleven normal horses in Experiment 1, there was no significant difference in the values obtained from paired carpal and tarsal joints. The wide

range of variation in the measurements obtained from each joint may reflect the fact that the population of animals sampled was heterogenous in relation to age and use, and also parallels the wide range of serum values reported in both normal humans (Thonar and others 1985) and horses (Alwan and others 1990). Hascall and Glant (1987) suggest that the extent and degree of sulphation may vary considerably from person to person, possibly accounting for the wide range of variation seen in serum samples. This may also be the case with equine synovial fluid and serum samples. Yovich and others (1991) found that synovial fluid KS values ranged from 62,200 ng/ml to 151,500 ng/ml, median 101,100 ng/ml in the midcarpal joint in a group of normal horses (n=19). Right and left midcarpal joints in these horses were not compared. In the current study, KS values for the midcarpal joints varied from 6,820 to 185,139 ng/ml, median 40,962 ng/ml, (right midcarpal joint) to 6, 293 to 267,216 ng/ml, median 38,870 ng/ml (left midcarpal joint). These values are not directly comparable to those of Yovich and others as the populations of animals were different, the criteria for assessing normality were dissimilar and different assays were used. The modified DMB assay measures all sulphated GAG, which differs from the immunoassay in the current study which is quantitating only the KS epitope recognised by the MAb.

In the study of Alwan and others (1990), carpal joint KS values ranged from 3,000 ng/ml to 80,000 ng/ml with a mean value of 20,800 ng/ml. Again, paired joint values were not assessed in the thirty normal joints sampled, nor were the carpal joints assayed specified in terms of the actual compartment, (radiocarpal or midcarpal joint), from which the fluid was obtained. Serum values of KS in normal horses ranged from 90 ng/ml to 6,800 ng/ml compared to 82 to 428 ng/ml in the current study. Again, it is difficult to draw comparisons between the actual values obtained as, although the format of the ELISA used is similar, the standards used as reference antigen differ from those used in the current study and the population of animals from which these samples are taken may differ in terms of criteria used to define "normality". However, irrespective of the methods used to measure KS, all studies described in horses generally seem to show that a wide range of values may be found in normal animals.

In the current study, there was an increase in 8 of the 11 horses in the level of KS measured in the left radiocarpal joint compared to the left midcarpal joint. It is difficult to explain why this is the case in this group of horses, as there was no evidence of e.g. acute articular cartilage injury or pathology that may have accounted for an elevation of KS in the synovial fluid of these animals. It was noted that three of these horses had values for the left radiocarpal joint that were more than double the values for the corresponding midcarpal joint and these animals may have biased the data in the small sample number used here. It would be interesting to evaluate whether this elevation in KS was present in a larger population of normal horses than those examined here.

2.4. Diurnal and day to day variation in serum KS levels

In the group of six ponies studied in Experiment 2, there was no evidence of diurnal or day to day variation in serum KS levels. This finding in horses was similar to that of Block and others (1989) wherein serum levels of KS in humans carrying out normal activity did not vary significantly during the course of a single day or from day to day over a period of one week. Analysis of mean KS levels comparing each time period did not reveal any significant changes in KS concentration during any specific time of day or day of the week in human subjects. This was also the case in the population of ponies studied here. The maximum temporal variation in any given pony in this group was 38 per cent in the day to day study and 44 per cent in the diurnal study. This was considerably higher than in the human study where the maximum temporal variation was 10 per cent, and this figure was used as the basis on which to state that a single KS measurement was representative of a stable and unchanging KS serum concentration. In the study of Block and others (1989), subjects were allowed to carry out moderate exercise, which may have affected the clearance rate of KS from synovial fluid to serum and possibly its metabolism. In the current study, the ponies were restricted to no exercise while samples were being collected and this may account for the difference in results compared to those of Block and others (1989).

It was observed in the current study that Pony No. 103 had serum KS levels that were significantly higher (e.g. 415 ± 36.7 ng/ml of KS equivalents in the diurnal variation study) compared to the other animals, e.g. Pony No. 104 (98 ± 28.5 ng/ml of KS equivalents). Pony No. 103 also had serum KS values in the day to day variation study that were markedly higher than the other animals in the group. Although these ponies had no history of joint disease and no evidence of joint disease on inspection, it may be possible that this animal had undetected joint disease. May (1993 unpublished) has stated that there may be an inherited biochemical abnormality of cartilage in horses, accounting for OA changes seen in unbroken animals as young as two years of age. This may be another possible explanation for the high serum KS levels in this animal. In the absence of OA, it may be likely that very high circulating levels of KS in this pony may be due to individual biologic variation, however, there is no data available currently on serum KS levels in a large group of young unbroken animals to determine if this is in fact the case.

2.5. Effects of exercise on serum KS levels

In the group of six horses studied in Experiment 3, there was a significant increase in serum KS levels post-exercise on the first exercise test. This increase was not observed on the second exercise test, in which only four of the original six animals were available for blood sampling. However, trends showed that there was a graphically evident rise in serum KS comparing pre and post-exercise values. It would be desirable to study a larger group of animals on another occasion to ascertain if the elevation in serum KS levels with exercise is repeatable. It has been reported in humans by Block and others (1988), that serum KS levels do not increase with moderate exercise and by Thonar and others (1991) that strenuous exercise, (i.e. running a marathon) does not result in a significant increase in serum KS levels. It was noted that the baseline serum KS values in these horses did not vary on the first and second sampling occasions, which were carried out on the same week day (two weeks apart) to eliminate a "day" effect. This showed that the levels of serum KS did not vary greatly from week to week in this study.

2.6. Studies in horses with osteoarthritis

In the study of Todhunter and others (1993), ponies undergoing surgery to create osteochondral defects in the radiocarpal joint were exercised at five days post-operatively, while on medication with PBZ. Plasma KS levels peaked two days after joint injury in all ponies and were lowest twenty one days after joint treatment. In two of the three cases of chronic joint disease (three to six months duration) described in the current study, serum KS levels were significantly elevated two days after surgery, which was similar to the findings of Todhunter and others (1993) and were starting to decrease by day four post-surgery (Case 2 and 3). With Case 1, serum KS levels were not elevated two days post-operatively, but started to decrease 5 days post-operatively. It is difficult to explain why this horse had an elevated synovial fluid and serum KS value on Day 0 compared to 3 days pre-surgery as there was no evidence of iatrogenic damage to the articular cartilage as a result of intra-articular anaesthesia at arthroscopy and the procedure had been carried out as atraumatically as possible. It is unlikely that the anaesthetic agent used (mepivicaine) had any adverse effect on the articular cartilage or synovial membrane that would result in a measurable increase in KS. This horse was monitored to 106 days post-operatively and serum KS levels remained reasonably consistent in the post-operative recovery period. The horses in this study were on a similar treatment regime with PBZ as those of Todhunter and others (1993). The pharmacokinetics of PBZ in equine synovial fluid and its effect on synovial metabolism is currently unknown. Similarly, the effect of PBZ on equine PG synthesis is also unknown. A potential exists for monitoring the effects of PBZ in conjunction with post-operative management programmes, using KS as a marker of articular cartilage metabolism.

CHAPTER VI

CONCLUSIONS

1. APPLICATION OF THE KERATAN SULPHATE ASSAY

Several studies (Thonar and others 1985; Carroll 1989; Sweet and others 1988) have suggested the measurement of serum KS has potential for diagnosis of OA in man. However, the accuracy of the assay relies on several factors including size and degree of sulphation of the KS epitopes in serum from OA patients (Caterson and others 1989) which may differ from KS epitopes in healthy subjects. It has been concluded from studies on humans with various arthritic disorders that estimations of KS have not proved to be true diagnostic assays as they only retrospectively confirm pre-existing diagnosis (Caterson and others 1989). As studies have shown (Ratcliffe and others 1988; Todhunter and others 1993), KS levels are elevated in the acute stages of joint injury compared to the more chronic stages. The level of KS in advanced OA therefore has little relevance to the performance horse. It only retrospectively confirms a diagnosis of OA which has already compromised the animal's athletic ability, at a stage in the disease process where treatment may not render the joint fully functional.

In the study of Alwan and others (1990) and Yovich and others (1991), there was no difference in KS levels measured from normal joints and joints with OA. However, the chronicity of the condition and the severity of clinical signs were not staged, so it is difficult to interpret these findings. Careful selection and definition of patient groups is an important factor in the meaningful interpretation of KS results in both human and equine studies. It is also important to bear in mind that the contribution of KS released with normal metabolism in the joint to the quantity of KS measured at any given sampling time in clinical cases of OA is unknown. Brandt (1991) voices this concern, however the issue has not, to date been addressed in human and veterinary studies. Development of a panel of monoclonal antibodies that identify anabolic markers synthesised in OA could potentially address this problem in a more satisfactory manner.

Tests such as the KS assay may provide information on the activity of disease and possibly on the prognosis and response to therapy, however, they do not, at this time, provide a diagnosis of the type of disease activity affecting the joint on their own (Todhunter and others 1993). If this assay is to be used to evaluate articular cartilage status in OA, it would be best to use it in conjunction with other biochemical markers as outlined in Chapter I. Using multivariate statistical analysis, a combination of these variables would be useful in providing a more accurate prognosis.

It would also be important to consider the effects of various assay methods on the interpretation of KS results in human and equine studies. With regard to equine assays, it would be advisable to standardise the ELISA method used as much as possible in terms of standard used to produce the standard curve and the MAb

used so that results could be meaningfully interpreted and referenced against those from other institutions. The assay used in the current study was essentially that of Thonar and others (1985), which is widely used in human medicine and results were reproducible from day to day. It may be advisable to use equine cartilage standards in the ELISA to measure KS in equine samples to reduce the possibility of species differences in KS affecting the recognition of epitope. It may also be useful to prepare equine standards in KS-depleted equine serum (using solid phase antiserum to adsorb KS) to reduce the possibility of a matrix effect.

2. POTENTIAL FUTURE APPLICATIONS OF KERATAN SULPHATE MEASUREMENT IN HORSES

A potential use for monitoring KS may arise in longitudinal blood sampling of racehorses during training to identify animals at possible risk of developing OA. The wide range of serum KS levels in the equine population in the current study, also reported by other workers, would suggest that in any future investigations, longitudinal studies, where each animal provides its own baseline values, should be carried out. It would be ideal to sample these animals prior to commencing a period of training in order to establish baseline values with which to compare results obtained throughout a subsequent work programme. It is known that the content of KS in articular cartilage increases with exercise (Jones, Torssan and Lamperg (1977). It would be important to establish a data base of longitudinal sampling results on young, unbroken Thoroughbred horses (not destined for racing) with which to compare data from unbroken racehorses of a similar age group that subsequently undergo a training programme in order to evaluate the effect of an exercise regime on KS levels compared to a control group. The current study has shown that there is no diurnal or day to day variation in serum KS levels in normal animals, and that as such, measurements of KS represent a steady state.

Monitoring serum KS may also be beneficial in attempting to elucidate the possible reasons for the appearance of OA in young unbroken animals, which has been suggested as an inherited biochemical abnormality. It would be important however, to have sufficient age matched "control" horse sampling data in order to differentiate possible early OA KS levels from age-related changes in serum KS which are known to occur in humans (Sommarin and Heinegard 1986; Thonar and others 1990) but which have, as yet, not been investigated in the horse.

3. FUTURE TRENDS IN PROTEOGLYCAN ASSAYS

Current research in human medicine is targeting on neoepitopes generated by individual enzymes involved in the PG degradation process. Efforts are being concentrated on the development of a panel of monoclonal antibodies specifically directed against the new N and C terminal domains generated by the degradation of cartilage generated by extracellular metalloproteinases. Metalloproteinases can be detected by immunoassay, but this does not take into account whether these enzymes are active, or the presence of inhibitors which neutralise their activity. The immunological detection of enzyme-derived epitopes is directly related to

the degree of specific involvement of individual proteases. Development of a panel of monoclonal antibodies could be used to detect the enzymes involved in cartilage destruction (Caterson 1991). Current research is also concentrating on anabolic markers found in newly synthesised PG which occur as a result of the chondrocyte's attempts to repair and remodel the connective tissue around it. In addition there are anabolic markers present at an early stage of the disease process before there is any significant loss of PG from the tissue or any evidence of cartilage destruction (Caterson, Mahmoodian, Sorrel and others 1990). These offer some potential for truly early diagnosis of OA preceding the onset of clinical signs.

LIST OF APPENDICES

APPENDIX 1 **Suppliers of reagents and materials**

Arnolds Veterinary Products Ltd., Shrewsbury, U.K.	Intraepicaine Equipalazone
A/S NUNC Kamstrupvej 90, Kamstrup DK-4000, Roskilde, Denmark.	ELISA plates.
Beckman Ltd., Progress Road, Sands Industrial Estate, High Wycombe, Bucks., U.K.	Centrifuge tubes and rotors
C-Vet Ltd., Minster House, Western Way, Bury St. Edmunds, U.K.	Phenyzene
Dynatech Laboratories Daux Road, Billingshurst, West Sussex, U.K.	Plate reader and computer software
ICN Biomedicals Ltd., Eagle House, Peregrine Business Park, Gomm Road, High Wycombe U.K.	Chondroitin ABC lyase 1/20/5-D4 monoclonal antibody Plate sealers Titertek pipette
MSE Manor Royal, Crawley, Sussex, U.K.	Tube piercer.

Minitab (Release 9 for Windows), Clecom Microcomputer Specialists, The Research Park, Vincent Drive, Edgbaston, Birmingham, U.K.	Minitab statististics package
Paar Scientific Ltd., 594 Kingston Road, Raynes Park, London. U.K.	Paar digital density meter.
Pierce and Warriner, Chester, U.K.	Guanidine hydrochloride (Sequanal grade)
Pierce U.K. Ltd. 36, Clifton Road, Cambridge, U.K.	Dialysis Membrane. Vol./cm. 7 ml. MW: 6000-8000.
Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, U.K.	General chemicals
Scotlabs., Wishaws Road, Coatbridge, Strathclyde, UK	Treff tubes
Scottish Antibody Production Unit. Law Hospital, Carluke, Lanarkshire, U.K.	SAM-HRP Anti-mouse Ig G.

APPENDIX 2.
Buffer composition for proteoglycan purification.

Buffer for cartilage.

TRIS BUFFER

Trisma base	0.05M	0.61 g/L.
Sodium chloride	0.15M	8.77 g/L.
EDTA	0.01M	3.72 g/L.
Sodium azide(w/v)	0.01per cent	0.10 g/L.

Adjust pH to 4.3

SODIUM ACETATE BUFFER
100 mls contains:

Sodium acetate trihydrate	0.05M	0.68 g.
6-amino caproic acid	0.10M	1.31 g.
EDTA	0.10M	0.37 g.
Benzamidine	0.005M	0.078 g.

7.5 M GUANIDINE HYDROCHLORIDE SOLUTION
100 mls contains:

Guanidine hydrochloride	7.5M	57.32 g.
EDTA	0.1M	0.37 g.
6-amino caproic acid	0.1M	1.31 g
Benzamidine	0.005M	0.078 g.

DIALYSIS SOLUTION
500 mls contains:

Sodium acetate	0.05M	3.40 g.
EDTA	0.10M	1.86 g.
6-amino caproic acid	0.10M	6.56 g.
Benzamidine	0.005M	0.39 g.

APPENDIX 3

Calculation of the theoretical gradient formed during associative and dissociative density gradient centrifugation.

$\frac{dp}{dr}$=density gradient at the position ro , the centre of the Gaussian band.

$$\frac{dp}{dr} = \frac{w^2 r}{B(p)} = (angular\ velocity)^2 \times density\ gradient\ at\ position\ ro$$

B= A function of the temperature, activity, molecular weight and partial specific volume of the solute at a density *p*.
B is evaluated by graphical methods from published data of densities (Ifft *et al,* 1961) and activity coefficients.

Associative Density Gradient calculations.

*p*o= 1.69 g /ml. Maximum rotor speed = 37, 500 rpm.

$$\frac{dp}{dr} = \frac{(37500)^2 \times 6.271 \times 0.010966}{1.190 \times 10^9}\ g/ml/cm$$

$$= 0.0813$$

Therefore, $p\ max.$= 1.69 + (0.0813x 1.938)

=1.85 g / ml.

w= angluar velocity.

Rotor type used: Beckman Ti 60.

$p\ min.$= 1.69- (0.0813x 2.17)

= 1.51 g / ml.

Dissociative Density Gradient calculations.

*p*o= 1.5 g /ml. Maximum rotor speed= 37, 500 rpm.

Therefore,

$$\frac{dp}{dr} = \frac{(37500)^2 \times 6.271 \times 0.010966}{1.245 \times 10^9} \; g/ml/cm$$

$$= 0.0777.$$

Therefore, *p max.*= 1.5 + (0.0777 x 1.938)

$$= \mathbf{1.65}$$

p min.= 1.5-(0.0777 x 2.17)

$$= \mathbf{1.33}$$

APPENDIX 4

Buffer preparation for plate coating

Chondroitin ABC lyase buffer.
Volume prepared = 100 mls.

Sodium acetate	0.1 M	0.22 g/100 mls.
Tris	0.1 M	1.21 g/100 mls.

Adjust pH to 7.3 using conc.HCl.

Plate coating buffer.
Volume prepared = 1 litre.

Sodium carbonate	20 mM	1.12 g/L.
Sodium bicarbonate	20 mM	1.68 g/L.
Sodium azide	0.002 %	20 mg/L.

Adjust pH to 9.2 using conc. HCL.

APPENDIX 5

Buffer for preparation of MAb stock .

PBS buffer for dilution of Mab to make a 1/100 dilution of MAb.

Per litre of MAb buffer:

Sodium chloride	120mM	54.55g.
Disodium hydrogen orthophosphate	5mM	0.71g.
EDTA	10mM	3.72g.
Trizma base		0.5 ml per Litre.

Adjust pH to 7.0 using conc.HCl.

APPENDIX 6. Checkerboard for titration of MAb and SAM-HRP

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	1/1600K **1/50**	1/1600K 1/100	1/1600K 1/200	1/1600K 1/400								
C MAb	1/800K **1/50**	1/800K **1/100**	1/800K **1/200**	1/800K **1/400**								
D	1/400K **1/50**	1/400K **1/100**	1/400K **1/200**	1/400K **1/400**								
E	1/200K **1/50**	1/200K **1/100**	1/200K **1/200**	1/200K **1/400**								
F	1/100K **1/50**	1/100K **1/100**	1/100K **1/200**	1/100K **1/200**								
G	1/50K **1/50**	1/50K **1/100**	1/50K **1/200**	1/50K **1/400**								
H	1/25K **1/50**	1/25K **1/100**	1/25K **1/200**	1/25K **1/400**								

MAb is vertically diluted out (in columns), **SAM-HRP** is diluted out in rows.

APPENDIX 7. Microtitre plate for determination of monoclonal antibody titration

	1	2	3	4	5	6	7	8	9	10	11	12
A	1/712	1/712	1/356000	1/356000	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	1/356	1/356	1/128000	1/128000	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
C	1/128	1/128	1/64000	1/64000	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
D	1/64	1/64	1/32000	1/32000	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
E	1/32	1/32	1/16000	1/16000	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
F	1/16	1/16	1/8000	1/8000	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
G	1/8	1/8	1/4000	1/4000	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
H	1/4 WS	1/4 WS	1/2000 MAb	1/2000 MAb	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

IRS= International reference standard, **MAb** = monoclonal antibody.

APPENDIX 8

JOINT NAMING CODE

Mark left limb joints as (L) and right limb joints as (R).

A= metacarpophalangeal joints.

B=radiocarpal joints.

C=intercarpal joints.

D=metatarsopophalangeal joints.

E=tibiotarsal joints.

SE=serum.

Example:

Horse No.1. (Number corresponds to the horse's owner).
Right fore radiocarpal joint=1BR.
Serum sample from No.1=1SE.

APPENDIX 9

Composition of buffers and reagents used in the KS ELISA.

Phosphate buffered saline used for sample, standard and antibody dilution and for plate washing.

PBS stock solution (10x)

Potassium chloride	0.026M	2g /litre.
Potassium dihydrogen phosphate	0.015M	2g /litre.
Sodium chloride	1.37M	80g /litre.
Disodium hydrogenorthophosphate (anhydrous).	0.08M	11.4g /litre.

Prepare a 1 in 10 dilution of the stock solution as required using distilled water.

Working buffer

Phosphate buffered saline , 0.05 per cent Tween 20 (0.5 ml of Tween-20 /L of PBS.)
Adjust pH to 5.3 using conc. HCl.

PBS-Tween 20, 1 per cent BSA, pH 5.3 (antibody buffer)

100 ml of PBS contains 1g of Bovine Serum Albumin (BSA).
This buffer is used for all antibody dilutions.
Volume made up=1 L.

Citrate / Phosphate buffer

Volume made up=200 ml.

Citric acid	0.05 M	1.05 g/100ml.
Disodium hydrogen orthophosphate	0.10 M.	1.42 g/100ml.

Components are made up in distilled water. 100 ml of citric acid is added to approximately 90 ml of disodium hydrogen orthophosphate and adjusted to pH 5.0 using the remainder of the disodium hydrogen orthophosphate. The buffer is stored at 4 °C.

OPD colour reagent

The colour reagent is made up immediately prior to use. The citrate phosphate buffer, stored at 4 °C is first allowed to come to room temperature.
To 25 ml of citrate / phosphate buffer add a 10 mg tablet of *o*-phenylene diamine and 11 µl of 30 per cent hydrogen peroxide. The colour reagent should be tested prior to use by adding a small amount to the SAM-HRP preparation and observing the colour change to orange-brown within 5 seconds.

APPENDIX 10. Keratan sulphate microtitre plate-intra-assay C.V. layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**1/712**	**1/712**	1/1280	1/1280	1/80	1/80	1/80	1/80	1/80	1/80	1/80	1/80
B	**1/356**	**1/356**	1/640	1/640	1/40	1/40	1140	1/40	1/40	1/40	1/40	1/40
C	**1/128**	**1/128**	1/320	1/320	1/20	1/20	1/20	1/20	1/20	1/20	1/20	1/20
D	**1/64**	**1/64**	1/160	1/160	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10
E	**1/32**	**1/32**	1/80	1/80	1/80	1/80	1/80	1/80	1/80	1/80	1/80	1/80
F	**1/16**	**1/16**	1/20	1/40	1/40	1/40	1/40	1/40	1/40	1/40	1/40	1/40
G	**1/8**	**1/8**	1/20	1/20	1/20	1/20	1/20	1/20	1/20	1/20	1/20	1/20
H	**IRS 1/4**	**IRS1/4**	QCS1/10	QCS1/10	QCS1/10	QCS1/10 -	QCS1/10	QCS1/10	QCS1/10	QCS1/10	QCS1/10	QCS1/10

IRS= International Reference Standard, **QCS** = Quality Control Serum.

APPENDIX 11

CASE INFORMATION (EXPERIMENT 1)

Horses classified as "normal"in the current study were those euthanased for reasons other than joint disease, with no history of joint disease and no evidence of lameness or joint distension on examination. All joints were opened and visually inspected at post-mortem examination. Cases were from the **Equine Clinic (Medicine) at Glasgow University Veterinary School,** except **No.11 (Royal (Dick) School of Veterinary Studies).**

No.	Case Source	Age (years)	Sex	Breed	Use	Cause of Death
1	**Medicine**	4	Mn	Clyde	UB	Grass sickness
2	**Medicine**	2	Mn	TBx	UB	Colic
3	**Medicine**	14	F	TBx	RIH	Squamous cell carcinoma
4	**Medicine**	4	F	Clyde	UB	Colitis
5	**Medicine**	7	Mn	TB	NHRH	Colic
6	**Medicine**	15	F	Pony	RIH	Chronic diarrhoea
7	**Medicine**	5	F	Pony	RIH	Grass sickness
8	**Medicine**	7	Mn	TBx	RIH	Grass sickness
9	**Medicine**	5	Mn	TB	FRH	Tendon injury (chronic)
10	**Medicine**	8	Mn	TBx	RIH	Colic
11	**R(D)SVS**	4	Mn	TB	HU	Colic

Mn=gelding, F=mare, TB=Thoroughbred, TBx=Thoroughbred cross, Clyde=Clydesdale, UB=unbroken, FRH=Flat racehorse, NHRH=National Hunt racehorse, RIH=Riding horse, Hu=Hunter.

APPENDIX 12

Normal horse joint and serum KS data
Results expressed as ng/ml of KS equivalents

NO.	BR	BL	CR	CL	ER	EL	SE
1	109,990	68,518	106,564	72,247	87,342	90,150	**163**
2	120,678	193,043	129,584	111,856	96,798	144,333	**254**
3	36,400	88,780	40,962	43,232	57,619	59,270	**428**
4	204,474	182,494	185,139	267,216	99,776	100,429	**332**
5	29,923	24,716	16,081	13,802	17,667	14,527	**209**
6	5,300	5,086	6,820	6,293	6,631	7,182	**150**
7	95,194	49,293	109,413	38,870	90,281	101,865	**183**
8	43,032	136,250	49,968	79,363	86,211	84,720	**182**
9	8,382	15,486	16,455	15,249	8,505	12,778	**82**
10	11,166	10,204	12,020	9,913	10,613	7,163	**140**
11	5,762	14,165	10,338	9,363	11,483	10,870	**246**

KEY: **B**= radiocarpal joint. **C**= intercarpal joint. **E**= tibiotarsal joint.
SE= serum.
R= right limb.
L= left limb.

APPENDIX 13

PONY INFORMATION (ANIMALS USED FOR DAY TO DAY AND DIURNAL VARIATION STUDIES).

All ponies were aged 15 to 16 months old. All were Shetland ponies.
Animals were housed with no exercise. These animals were being used as part of another study, under Home Office licence, and "leftover" serum from that experiment was used in the current study.

Pony No.	Sex
101	M
102	F
103	M
104	F
105	F
106	F

APPENDIX 14

Racehorse data

Horse No.	Breed	Age	Sex	Use
1	TB	10	MN	FRH
2	TB	2	F	FRH
3	TB	3	F	FRH
4	TB	2	MN	FRH
5	TB	3	MN	FRH
6	TB	3	F	FRH

TB= Thoroughbred, F= Mare, Mn= Gelding, FRH= Flat Racehorse.

GLOSSARY

A1D1	Associative1, Dissociative 1.
ADGC	Associative Density Gradient Centrifugation.
CS	Chondroitin Sulphate.
CLYDE	Clydesdale.
C.V.	Coefficient of Variation.
DDGC	Dissociative Density Gradient Centrifugation.
DJD	Degenerative Joint Disease
DMB	Dimethylene Blue.
ELISA	Enzyme Linked Immunosorbent Assay.
ESE	Equine Serum.
ESF	Equine Synovial Fluid.
F	Female.
FRH	Flat Racehorse.
GAG	Glycosaminoglycan.
HSE	Human Serum.
HSF	Human Synovial Fluid.
HU	Hunter.
IL-1	Interleukin-1.
IRS	International Reference Standard.
KS	Keratan Sulphate.
M	Colt.

MAb	Monoclonal Antibody.
mins	minutes.
ml	mililitres.
MN	Gelding.
ng	Nanograms.
NHRH	National Hunt Racehorse.
OA	Osteoarthritis.
OCD	Osteochondrosis.
OD	Optical Density.
OPD	*O*-phenylene diamine.
PBS	Phosphate Buffered Saline.
PBZ	Phenylbutazone.
PG	Proteoglycan.
PAI-1	Plasminogen Activator Inhibitor-1.
PSGAG	Polysulphated Glycosaminoglycan.
QCS	Quality Control Serum.
RIH	Riding Horse.
SAM-HRP	Sheep Anti-Mouse IgG Horseradish Peroxidase.
TB	Thoroughbred.
TBx	Thoroughbred cross.
TGF ß	Transforming Growth Factor Beta.
TNF	Tumour Necrosis Factor.
UB	Unbroken horse.

WS Working Standard.

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