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**The dose related effects of phenylbutazone and a methylprednisolone acetate formulation (Depo-Medrol®) on cultured explants of equine carpal articular cartilage.**

A thesis presented in partial fulfilment of the requirements

for the degree

**of Master of Veterinary Science  
in Veterinary Pharmacology and Toxicology  
at Massey University.**

**William Thomas Jolly**

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## ABSTRACT

Experimental methods involving the maintenance of explants of equine articular cartilage in tissue culture, an amino sugar assay, radiolabelling, and histology were developed and validated.

The dose related effects of phenylbutazone and Depo-Medrol® on chondrocyte viability and chondrocyte mediated synthesis and depletion of proteoglycans were investigated using cultured explants of equine middle carpal joint articular cartilage. Explants from 12 horses (941 x 3 mm diameter) were cultured for a total of 5 days, which included 3 days exposure to either phenylbutazone (0, 2, 20, 200, 2000  $\mu\text{g mL}^{-1}$ ), or Depo-Medrol (0, 20, 200, or 2000  $\mu\text{g mL}^{-1}$ ). For each explant, amino sugar content was used as a measure of proteoglycan content,  $^{35}\text{S}$  incorporation as a measure of the rate of proteoglycan synthesis, and the number of pyknotic nuclei as a measure of cell death.

During culture, control explants remained metabolically active and viable but suffered a net loss of proteoglycans. Proteoglycan loss was reduced by the presence of either phenylbutazone or Depo-Medrol. This effect was significant at clinically relevant concentrations of phenylbutazone (2-20  $\mu\text{g mL}^{-1}$ ), but not Depo-Medrol (20-200  $\mu\text{g mL}^{-1}$ ). Depo-Medrol caused a dose-dependent suppression of proteoglycan synthesis at all concentrations, but chondrocyte viability was affected at only the 2000  $\mu\text{g mL}^{-1}$  dose. Phenylbutazone affected proteoglycan synthesis and cell viability at only the 2000  $\mu\text{g mL}^{-1}$  concentration. At all concentrations, the anti-catabolic effects of each drug influenced the proteoglycan content of the explants far more than did any anti-anabolic or cytotoxic drug effect.

The results suggest that the therapeutic potential of both phenylbutazone and Depo-Medrol may not be just restricted to their anti-inflammatory effects on the soft tissues of the joint, but may also involve a suppression of the synthesis and/or activation of proteolytic enzymes within the cartilage itself.

## PREFACE

Lameness has been reported as the number one cause of lost training days and failure to race in the thoroughbred industry (Jeffcott *et al.*, 1982; Rossdale *et al.*, 1985). Joint associated lameness accounted for a third of the lamenesses localised. Causes of joint lameness include soft tissue inflammations, infections, osteochondritis disicans, degenerative joint disease, ligamental problems and intra-articular fractures. All of the above conditions may progress to degenerative joint disease.

Corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat degenerative joint disease (osteoarthritis) in animals and man. Degenerative joint disease is characterised by deterioration of the articular cartilage, accompanied by changes to the bone and soft tissues of the joint (McIlwraith, 1982). Treatment aims include resolution of initiating causes, restoration of function, and prevention of further articular cartilage damage (McIlwraith & Vachon, 1988). Because the reparative response of articular cartilage is inadequate (Desjardins & Hurtig, 1990), loss of articular cartilage often limits the complete restoration of athletic function (Bramlage *et al.*, 1988; Richardson & Clark, 1991).

The pathogenesis of degenerative joint disease is incompletely understood (McIlwraith & Vachon, 1988). However, the release of proteoglycans is recognised as one of the earliest responses of articular cartilage to injury (Mankin, 1974; Clyne, 1987). It has been proposed that proteoglycan depletion resulting from increased proteoglycan catabolism may leave the chondrocytes and the collagen structural framework more susceptible to further mechanical damage and thus perpetuate the cycle of degeneration (Harris *et al.*, 1972; McIlwraith & Van Sickle, 1981). The relative significance of chondrocyte mediated proteoglycan catabolism versus that mediated by enzymes released from the synoviocytes and migrant leucocytes has not been established (Fell & Jubb, 1977; McIlwraith & Van Sickle, 1981; Martel-Pelletier *et al.*, 1984; Hurtig, 1988; McIlwraith & Vachon, 1988; May *et al.*, 1991). Cytokine and drug induced

suppression of proteoglycan synthesis may also contribute to the proteoglycan depletion in some osteoarthritic conditions (Palmoski & Brandt, 1983; MacDonald *et al.*, 1992; May *et al.*, 1992).

Methylprednisolone acetate (MPA) and phenylbutazone (PBZ) are the most common steroid and non-steroidal anti-inflammatory drugs used for treatment of joint injury in equine athletes. Their soft tissue mediated clinical effects are well recognised (Higgins & Lees, 1984). Whether or not they also confer some degree of chondroprotection is actively debated (Tobin *et al.*, 1986; Burkhardt & Ghosh, 1987; McIlwraith, 1989). Furthermore, there is some evidence to suggest their use may actually potentiate the progression of joint deterioration (Whitehouse & Bostrum, 1962; Tobin *et al.*, 1986; Chunekamrai *et al.*, 1989; Trotter *et al.*, 1991; Shoemaker *et al.*, 1992). Both the types and mechanisms of their effects on articular cartilage are subjects of some conjecture and much controversy (May *et al.*, 1987; McIlwraith & Vachon, 1988; Saari *et al.*, 1992).

Relatively few controlled *in vivo* trials have sought to investigate the effects of MPA or PBZ on equine articular cartilage. Interpretation of specific drug effects from these trials has been hindered by their small sample numbers, the types of investigative procedures performed, and a range of confounding variables. The *in vitro* maintenance of tissue allows for a more controlled environment in which the study of specific interactions can be isolated from confounding variables (Tyler *et al.*, 1982).

The purpose of this study was to investigate the dose related effects of phenylbutazone, and a methylprednisolone acetate formulation (Depo-Medrol®)<sup>1</sup>, on chondrocyte viability, and chondrocyte mediated degradation and synthesis of matrix proteoglycans so as to better understand how these drugs exert their effects *in vivo*. The following three hypotheses were tested with respect to each of these parameters; (1) the drug is capable of affecting the parameter, (2) the effect is apparent at clinically relevant concentrations, and (3) the effect is greater at higher concentrations.

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<sup>1</sup> Depo-Medrol, Upjohn Inter-American Corporation.

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# TABLE OF CONTENTS

<b>ABSTRACT</b>	ii
<b>PREFACE</b>	iii
<b>ACKNOWLEDGEMENTS</b>	v
<b>LIST OF FIGURES</b>	xiii
<b>LIST OF TABLES</b>	xviii

## INTRODUCTION

### A. Articular cartilage structure and function

1. The chondrocytes . . . . . 2
2. Collagen 4
3. The proteoglycans . . . . . 5
4. Measures of matrix biosynthesis and depletion . . . . . 7
5. Response to injury . . . . . 11

### B. Use of intra-articular corticosteroids in the horse

1. Anti-inflammatory mechanism of action . . . . . 14
2. Manufacturer recommendations . . . . . 18
3. Pharmacokinetics . . . . . 19
4. Clinical use . . . . . 21
5. Adverse effects on joints . . . . . 22

### C. Effects of corticosteroids on articular cartilage

1. Normal articular cartilage . . . . . 24
  - (i) Chondrocyte morphology . . . . . 25
  - (ii) Matrix biosynthesis and depletion;  
*in vivo* results . . . . . 25
  - (iii) Matrix biosynthesis and depletion;  
*in vitro* results . . . . . 27
2. Articular cartilage of arthritic joints . . . . . 28
3. Articular cartilage repair . . . . . 29

**D. Use of phenylbutazone in the horse**

1.	Molecular mechanism of action	31
2.	Manufacturer recommendations	34
3.	Pharmacokinetics	
	(i) Absorption	35
	(ii) Distribution	35
	(iii) Metabolism and elimination	37
	(iv) Oxyphenbutazone kinetics	38
4.	Clinical use	39
5.	Adverse effects on joints	40

**E. Effect of phenylbutazone on articular cartilage**

.....	40
-------	----

**F. Summary**

.....	42
-------	----

**G. Objectives**

.....	43
-------	----

**H. Hypotheses**

.....	43
-------	----

## MATERIALS AND METHODS

### SECTION I: DEVELOPMENT OF THE MODEL

#### A. Analytical techniques

1. Biochemical analyses ..... 44
2. Scintillation counting ..... 54
3. DNA assay ..... 64

#### B. Measurement of proteoglycans in the culture media

1. Factors affecting the amino sugar assay ..... 67
2. Methods to reduce media and drug interference ..... 72
3. Separation of chondroitin sulphate from solutions  
containing serum proteins ..... 79
4. Separation of proteoglycans from solutions containing  
serum proteins ..... 89
5. Summary ..... 93

#### C. Explant culture characteristics

1. Variation in the amino sugar content and rate of  $^{35}\text{S}$   
incorporation between explants from different sites ..... 96
2. Viability of chondrocytes in cultured explants of  
equine articular cartilage ..... 99
3. Variation in the biosynthetic rate and total amino sugar  
concentration relative to time cultured ..... 106

## SECTION II: USE OF THE MODEL

### A. Explant Culture

1. Source of articular cartilage . . . . . 111
2. Preparation of cartilage samples . . . . . 111
3. Methylprednisolone acetate trial . . . . . 114
4. Phenylbutazone trial . . . . . 115

### B. Post-culture processing

1. Explants from the radial carpal bones . . . . . 115
2. Explants from the third carpal bones . . . . . 116

### C. Analytical techniques

1. Amino sugar assay . . . . . 116
2. Scintillation counting procedure . . . . . 116
3. Histological evaluation . . . . . 117

### D. Statistical methods

1. Explants from the radial carpal bones . . . . . 117
2. Explants from the third carpal bones . . . . . 117

## RESULTS

### A. Depo-Medrol (MPA) trial

1. Effect of Depo-Medrol on amino sugar content . . . . . 119
2. Effect of Depo-Medrol on <sup>35</sup>S incorporation . . . . . 119
3. Effect of Depo-Medrol on chondrocyte viability . . . . . 122

### B. Phenylbutazone (PBZ) trial

1. Effect of PBZ on amino sugar content . . . . . 124
2. Effect of PBZ on <sup>35</sup>S incorporation . . . . . 124
3. Effect of PBZ on chondrocyte viability . . . . . 127

### C. Histological trial observations

. . . . . 129

## **DISCUSSION**

<b>A. Discussion of the methods</b>	
.....	134
<b>B. Discussion of the results</b>	
.....	140
<b>C. Summary and conclusions</b>	
.....	148

## **APPENDIX**

<b>A. Tables</b>	
.....	150
<b>B. Statistical analyses</b>	
.....	161

## **BIBLIOGRAPHY**

.....	170
-------	-----

## FIGURES

2.1	Comparison of the absorption spectra of glucosamine and galactosamine (from 5-20 $\mu\text{g}$ ) . . . . .	47
2.2	Comparison of the absorption spectra of equivalent amounts of glucosamine, galactosamine, and chondroitin sulphate . . . . .	47
2.3	Absorption spectra of digested cartilage . . . . .	49
2.4	Comparison of the standard curves of glucosamine, galactosamine, and chondroitin sulphate . . . . .	50
2.5	The gradation of colours produced by a range of glucosamine amounts (2.5-20 $\mu\text{g}$ ) after being assayed according to the method of Gatt & Berman. The cuvette containing the yellow/orange solution is an example of the sensitivity of the assay to any variation in acidity . . . . .	51
2.6	Stability of the amino sugar assay over time . . . . .	53
2.7	Custom made cartilage chisel . . . . .	55
2.8	Cartilage punches . . . . .	56
2.9	Quench curve generated by $^{14}\text{C}$ quenched standards . . . . .	60
2.10	The effect of different wash procedures on the non-bound $^{35}\text{S}$ content of explants of equine articular cartilage . . . . .	62
2.11	Standard curve for calf thymus DNA assayed according to the method of Labarca and Paigen (1980) . . . . .	66

<b>2.12</b>	Absorption spectra of glucosamine standards dissolved in water . . .	68
<b>2.13</b>	Absorption spectra of glucosamine standards dissolved in DMEM . .	68
<b>2.14</b>	Absorption spectra of glucosamine standards dissolved in DMEM and assayed after oven digestion in 2N HCl . . . . .	68
<b>2.15</b>	Absorption spectra produced by DMEM, aqueous phenol red, and a 25% aqueous DMEM solution in response to the Gatt & Berman (1966) amino sugar assay (relative to a H <sub>2</sub> O blank, A = 0.5) . . . . .	74
<b>2.16</b>	Absorption spectra of chondroitin sulphate standards dissolved in DMEM following dialysis and oven digestion in 2N HCl (A = 2.0) . . . . .	74
<b>2.17</b>	Absorption spectra of DMEM and gentamicin following dialysis and oven digestion in 2N HCl (relative to a H <sub>2</sub> O blank, A = 2.0) . . . . .	77
<b>2.18</b>	Comparison of chondroitin sulphate standards dissolved in water and DMEM after dialysis and oven digestion . . . . .	78
<b>2.19</b>	The absorption spectrum produced by a crude papain solution . . . . .	81
<b>2.20</b>	Comparison of the absorption spectrum of glucosamine, and a glucosamine + papain combination . . . . .	83
<b>2.21</b>	Effect of papain on the assay of glucosamine standards . . . . .	84
<b>2.22</b>	Effect of serum protein precipitation on the amino sugar concentration of chondroitin sulphate standards . . . . .	87

<b>2.23</b>	Effect of serum protein precipitation and dialysis on the absorption spectra of aqueous chondroitin sulphate standards . . . . .	<b>88</b>
<b>2.24</b>	Effect of serum protein precipitation and dialysis on the absorption spectra of chondroitin sulphate standards dissolved in DMEM . . . . .	<b>88</b>
<b>2.25</b>	Effect of culture duration on the viability of chondrocytes within explants of equine articular cartilage . . . . .	<b>102</b>
<b>2.26</b>	Photomicrograph showing cell death at the edge of a cultured explant of equine carpal articular cartilage . . . . .	<b>103</b>
<b>2.27</b>	Photomicrograph showing cell death at the articular edge of a cultured explant of equine carpal articular cartilage . . . . .	<b>104</b>
<b>2.28</b>	Photomicrograph showing cell vacuolation in a cultured explant of equine carpal articular cartilage . . . . .	<b>105</b>
<b>2.29</b>	Effect of culture duration on the amino sugar content of explants relative to their initial wet weight . . . . .	<b>108</b>
<b>2.30</b>	Effect of culture duration on the amino sugar content of explants relative to their final DNA content . . . . .	<b>108</b>
<b>2.31</b>	Effect of culture duration on <sup>35</sup> S incorporation by explants relative to their initial wet weight . . . . .	<b>109</b>
<b>2.32</b>	Effect of culture duration on <sup>35</sup> S incorporation by explants relative to their final DNA content . . . . .	<b>109</b>

3.1	Photograph of an open equine middle carpal joint showing the sites (top = third carpal bone, bottom = radial carpal bone) where the strips of articular cartilage were harvested from . . . . .	112
3.2	Strip of articular cartilage from which explants have been cut . . . . .	113
3.3	Explants suspended in chilled DMEM awaiting further processing . . . . .	113
4.1	Effect of Depo-Medrol on the amino sugar content of cultured explants of equine middle carpal joint articular cartilage . . . . .	120
4.2	Effect of Depo-Medrol on <sup>35</sup> S incorporation by cultured explants of equine middle carpal joint articular cartilage . . . . .	121
4.3	Effect of Depo-Medrol on the viability of chondrocytes within cultured explants of equine middle carpal joint articular cartilage . . . . .	123
4.4	Effect of phenylbutazone on the amino sugar content of cultured explants of equine middle carpal joint articular cartilage . . . . .	125
4.5	Effect of phenylbutazone on <sup>35</sup> S incorporation by cultured explants of equine middle carpal joint articular cartilage . . . . .	126
4.6	Effect of phenylbutazone on the viability of chondrocytes within cultured explants of equine middle carpal joint articular cartilage . . . . .	128
4.7	Photomicrograph of an empty lacunae with the displaced nucleus sitting adjacent (1000x magnification) . . . . .	130

<b>4.8</b>	Photomicrograph of two dead chondrocytes close to the articular surface (4000x magnification) . . . . .	131
<b>4.9</b>	Photomicrograph of a chondrocyte cluster (4000x magnification) . . . . .	132
<b>4.10</b>	Comparison of the variation in proteoglycan content, as shown by alcian blue staining, of two sections cut from non-cultured explants harvested from the radial carpal bone of the same horse (400x magnification) . . . . .	133

## TABLES

2.1	DNA assay of digested cartilage samples . . . . .	65
2.2	Chemical composition of Dulbecco's modified Eagle medium (DMEM) . . . . .	70
2.3	Amino sugar assay absorbances following the addition of TCA and dialysis (524 nm) . . . . .	90
2.4	Amino sugar assay absorbances following the addition of NH <sub>4</sub> SO <sub>4</sub> and dialysis (524 nm) . . . . .	93
2.5	Variability in the amino sugar content (AS) of cultured explants from different carpal bones (μg mg <sup>-1</sup> ) . . . . .	98
2.6	Variability in the incorporation of <sup>35</sup> S by cultured explants from different carpal bones (dpm mg <sup>-1</sup> ) . . . . .	98
2.7	Chondrocyte death relative to time cultured (dead/total) . . . . .	101

## INTRODUCTION

### A. ARTICULAR CARTILAGE STRUCTURE AND FUNCTION

Articular cartilage is considered to be a hyperhydrated tissue having a water content of between 65 and 85% (Maroudas *et al.*, 1969; Mankin, 1974). It consists of a relatively sparse population of chondrocytes suspended in an extracellular matrix of collagen fibres and proteoglycan aggregates. Articular cartilage provides an almost frictionless articulating interface between adjacent bones while absorbing and distributing concussive, compressive and shearing forces.

The matrix, which is synthesized by the chondrocytes, is wholly responsible for the biomechanical properties of the tissue. The structure, composition, and cellularity of articular cartilage at any one site reflects the types and magnitudes of the biomechanical forces to which that area is subjected (Bramlage *et al.*, 1988). Thus, differences exist between different joints, sites within a given joint, between species, with age, and with injury (McIlwraith & Van Sickle, 1981; McIlwraith & Vachon, 1988; Vachon *et al.*, 1990; Richardson & Clark, 1990; Richardson & Clark, 1991). Loss of matrix, through enzymatic digestion or inhibition of synthesis, compromises the functional capacity of articular cartilage and can predispose the joint to degenerative joint disease (osteoarthritis). (McIlwraith & Vachon, 1988; Bramlage *et al.*, 1988).

Much of the information pertaining to the composition of equine articular cartilage has come either from histochemical studies, or has been inferred from research in other species. Only two studies have biochemically quantified some of the components of normal equine articular cartilage. Amino sugar and hydroxyproline concentrations were assayed as measures of the proteoglycan and collagen contents. Amino sugar concentrations differed widely between studies, between horses, between joints, and between sites within a given joint. The calculated average glycosaminoglycan content (dry weight) for one sampling site common to both studies varied from 7.3% (Vachon *et al.*, 1990) to 12% (Richardson & Clark, 1990). Collagen content was assessed by the first of these studies only (55.6% dry matter). Human articular cartilage has been

reported to have a dry weight composition of 60% collagen, 30% proteoglycans (approximately 90% of which are glycosaminoglycans), and 10% glycoproteins, lipids and chondrocytes (Mankin, 1974).

Histological techniques have been used in many anatomical and comparative studies. However, some confusion exists over the terminology used to describe the appearance of chondrocytes and matrix in histological sections. The term lacuna originated purely as a histological descriptive term and referred to an area which normally contained one or more chondrocytes surrounded by 'finely textured pericellular matrix' and into which 'fibrous matrix' did not encroach (Stockwell, 1979). Pericellular matrix usually refers to the matrix immediately surrounding the chondrocyte within the 'rim' of the lacuna, but is also sometimes used synonymously with territorial matrix. Territorial matrix describes the broad circular basophilic zone radiating out from many of the chondrocytes, while the inter-territorial matrix is basically the rest. Chondrones and chondrocyte clusters are also both histologically descriptive terms used to describe closely associated groups of chondrocytes which are thought to have resulted from relatively recent mitotic episodes. In contrast, the term chondron describes a physically isolatable structure consisting of a felt-like pericellular capsule constraining one or more chondrocytes and a quantity of pericellular matrix (Poole *et al.*, 1988).

## 1. The chondrocytes

The chondrocytes synthesize and maintain matrix structure and composition. Chondrocyte shape, size and density vary with distance from the articular surface, location within the joint, maturity, and joint pathology (Stockwell, 1979; Oikawa *et al.*, 1989; McIlwraith & Van Sickle, 1981). Chondrocyte density and metabolic activity are greatest in areas of the joint exposed to regular weight bearing (Palmoski *et al.*, 1980).

Four zones are recognised in adult articular cartilage. The **tangential zone**, or **superficial zone**, exists immediately below the articular surface and contains flattened chondrocytes. Next is the **transitional zone** which contains randomly distributed rounder chondrocytes. The third zone is the **radiate zone** where the chondrocytes have

a similar appearance to those in the transitional zone, but are arranged in short irregular columns. The deepest is the **calcified zone**, containing chondrocytes surrounded by hydroxyapatite, and separated from the radiate zone by the tideline (Weiss, 1968; Lust *et al.*, 1972).

The chondrocytes of the transitional and radiate zones are retained within 'felt-like' pericellular capsules (Poole *et al.*, 1988). These fibrous capsules can contain one or several chondrocytes. The capsule, the chondrocytes and the pericellular matrix are together referred to as a chondron. Frayed collagen fibres radiate from these chondrons and suggest a shear resistant, structural interrelationship between the capsular components and the type II collagen fibres of the matrix. The pericellular microenvironment contained within the chondron's fibrous capsule is thought to be involved in the regulation of chondrocyte metabolism (Poole *et al.*, 1988).

Mankin (1974) proposed that the chondrocytes of immature articular cartilage actively proliferate, but at a rate which dramatically decreases as the animal grows and matures. The chondrocytes of normal mature cartilage are essentially amitotic. As in other animals, the articular cartilage of neonatal foals exists as part of an articular-epiphyseal cartilage complex, which is thick in the neonate and decreases as endochondral ossification proceeds (Firth & Greydanus, 1987). In a study examining age-related changes in the thickness of the articular cartilage of equine third metacarpal bones, Oikawa *et al.* (1989) found there was no apparent histological pattern to the chondrocyte distribution in foals for their first 6 months of life. However, from approximately 6 months of age onwards, the more superficial chondrocytes were arranged parallel to the articular surface, and the middle and deeper chondrocytes existed in a more perpendicular type of pattern. As the yearlings matured the demarcation of calcified cartilage from non-calcified cartilage became evident as a distinct border, referred to as the tideline. At 19 months of age the chondrocyte distribution was characterised as being organised into the four classical zones, and after 24 months of age little further change in thickness was seen (Oikawa *et al.*, 1989).

The molecular mechanisms regulating chondrocyte replication are unknown (Tyler, 1985; Hickery *et al.*, 1990). There are no studies on changes in equine chondrocyte mitotic rate with age, or on the reasons for normal articular chondrocytes being essentially amitotic. However, chondrocytes within mature equine cartilage still retain a limited ability to replicate when exposed to adverse conditions (Riddle, 1970; McIlwraith & Van Sickle, 1981; Glade, 1990).

Adult articular cartilage is avascular, aneural, and devoid of lymphatics. Supply of nutrients and exchange of metabolic byproducts is by simple diffusion across the matrix to and from the synovial fluid and subchondral bone vasculature. This is aided by the movement of the fluid component of articular cartilage caused by the intermittent compression associated with weight bearing and joint movement (Maroudas & Evans, 1974; Pamoski *et al.*, 1980).

The chondrocytes of the tangential zone contain few intracytoplasmic filaments or inclusion bodies, whereas the chondrocytes of the transitional and radiate zone contain greater amounts of mitochondria, rough endoplasmic reticulum and golgi complex, indicative of greater metabolic activity (Lust *et al.*, 1972). The metabolic, catabolic, and biosynthetic activity of each chondrocyte is sensitive to its immediate extracellular oxygen tension, acidity, metabolite and nutrient concentrations (May *et al.*, 1991). The surrounding matrix composition, the depth from the articular surface, and the compression loading dynamics are thus all important determinants of the metabolic functioning of individual chondrocytes (Maroudas *et al.*, 1969; Maroudas & Evans, 1974; Handley & Lowther, 1977; Pamoski *et al.*, 1980; Sandy *et al.*, 1980; Jones *et al.*, 1982; Schneiderman *et al.*, 1986; Gray & Gottlieb, 1983).

## 2. Collagen

The collagen fibrils in articular cartilage interact to form a woven mesh restraining the proteoglycan aggregates, and providing tensile strength and resistance to shearing forces (Hickery *et al.*, 1990). Collagen strength depends on the formation of covalent bonds between its polymerized collagen molecules, but heterotypic cross-linking with other

protein types is also important to the stability of the resultant network (Broom, 1982). Type 2 collagen fibrils predominate in adult equine articular cartilage (Vachon *et al.*, 1990). The number, size and orientation of these fibrils vary with the depth of the articular cartilage. Classically (Benninghoff, 1925) they were described as being closely packed and arranged parallel to the articular surface superficially, and more widely spaced and more perpendicular to the articular surface with increasing depth. The alignment of individual collagen fibrils has now been shown to be somewhat more random, with distribution surrounding chondrocytes and in the superficial layer being the most ordered (Stockwell, 1979; Poole *et al.*, 1988). However, the resulting complex structural framework is responsible for restraint of the aggregated proteoglycans, and reflects the biomechanical conditions prevailing in any one area (Riddle, 1970; Lust *et al.*, 1972; Heinegard *et al.*, 1987; McIlwraith & Vachon, 1988; Desjardins & Hurtig, 1990).

### **3. The proteoglycans**

Proteoglycans are high molecular weight molecules (1000-3000 kDa) consisting of a central protein core (200-300 Kda) to which a large number of glycosaminoglycan side chains are covalently bound. Most of the proteoglycans contained within the collagen framework of articular cartilage are bound non-covalently to strands of hyaluronic acid, and stabilised with link proteins, to form large proteoglycan aggregates (Stockwell, 1979; Hardingham *et al.*, 1990). However, some are also firmly associated with the collagen fibrils (Sandy *et al.*, 1978; Hascal & Kimura, 1982; Nakano *et al.*, 1985). The physical size and steric conformation of the resulting aggregates are largely responsible for the ability of the collagen mesh to restrain the proteoglycan content of the matrix.

Up to 90% of the total mass of the proteoglycan aggregates are glycosaminoglycans, which are carbohydrate polymers (4-30 Kda) consisting of repeating disaccharide units. Glycosaminoglycan concentrations vary between individuals, between different joints, between sites within joints, with distance from the articular surface, and with the existence of any joint disease (Jones *et al.*, 1977; Schneiderman *et al.*, 1986; Richardson & Clark, 1990). Keratansulphate and chondroitin sulphate are the main

glycosaminoglycans of articular cartilage. Keratansulphate side chains tend to be concentrated towards the hyaluronic acid binding end, and chondroitin sulphate side chains predominate on the peripheral two thirds of the protein core (Hascal & Kimura, 1982). The relative proportions of keratansulphate and chondroitin sulphate can also vary between individuals, between joints, within joints, and with distance from the articular surface (Maroudas *et al.*, 1969; Jones *et al.*, 1977).

Keratansulphate consists of a repeating galactose and N-acetylglucosamine disaccharide unit. The glucosamine residue in the keratansulphate molecule is normally sulphated at the sixth carbon atom, but this sulphate group may be absent. Alternatively an extra sulphate group may be present at the sixth carbon atom of the galactose residue (Stockwell, 1979). Most disaccharide units contribute just one negative charge.

Chondroitin sulphate consists of a repeating glucuronic acid and N-acetylgalactosamine disaccharide unit. Chondroitin sulphate can exist as either chondroitin-6-sulphate or chondroitin-4-sulphate, depending on whether the sulphate group is bound to the sixth carbon atom or the fourth carbon atom of the galactose molecule. The proportion of chondroitin-6-sulphate increases with age. The length of chondroitin sulphate chains varies, and may consist of up to 100 disaccharide units. Generally each disaccharide unit is considered to contain one carboxyl and one sulphate group and contribute two negative charges, but there is good evidence to suggest mammalian chondroitin sulphate molecules are 'undersulphated' at the protein binding end (Wasteson & Lindahl, 1971). In contrast, shark chondroitin sulphate, often used as a reference standard, tend to have additional sulphate groups along its length and may average more than two negative charges per disaccharide unit (Stockwell, 1979).

The sulphate and carboxyl groups on the glycosaminoglycan side-chains result in the highly polyanionic nature of the matrix. The mutual repulsion of these negative charges dictates the steric conformation of the proteoglycan aggregates. Furthermore, the restraint of these highly polyanionic aggregates attracts a variety of cations, and the consequent large osmotic potential accounts for the high water content of cartilage. Cartilage is thus considered to be a biphasic material with a fluid phase which is readily

interchangeable with components of the synovial fluid. Fluid movement occurs by simple osmotic diffusion, or when the cartilage is subjected to compression. The restraint of these aggregated proteoglycans with their associated moveable water molecules results in the compressive stiffness, load distributing and friction minimising properties of articular cartilage. The balance between the expansive forces associated with the proteoglycans and the restraining tension in the collagen network, results in the compressive stiffness of the articular cartilage (Hardingham *et al.*, 1990). A change in either the proteoglycan content or structural integrity of the collagen framework can thus alter the functional capacity of articular cartilage (Harris *et al.*, 1972; Glade *et al.*, 1983; McIlwraith & Vachon, 1988)

Proteoglycan levels in adult articular cartilage are maintained by a continuous synthesis and turnover by the chondrocytes (Hickery *et al.*, 1990). In contrast to the rate of synthesis of collagen, the turnover of proteoglycans is considered to be relatively rapid, but estimates of the 'normal' rate of turnover vary widely (Maroudas & Evans, 1974). The activity of the chondrocyte, whether anabolic or catabolic, is influenced by the conditions immediately surrounding it. Physicochemical factors such as matrix hydration, osmotic pressure, pH, mechanical loading, and hormone and/or metabolic gradients are likely to be important (Schneiderman, *et al.*, 1986; Poole *et al.*, 1988).

The rate of glycosaminoglycan synthesis has been shown to vary inversely with local glycosaminoglycan concentrations (Maroudas & Evans, 1974; Handley & Lowther, 1976; Sandy *et al.*, 1978; Sandy *et al.*, 1980). Rates of synthesis also vary between species, individuals, ages, joints, normal and damaged joints, sites within joints, and with methods of quantification (Maroudas & Evans, 1974; Sandy *et al.*, 1980; Richardson & Clark, 1991).

#### **4. Measures of matrix biosynthesis and depletion**

Depletion of proteoglycan content results from imbalance of the normal turnover of proteoglycans, and can be due to a relative increase in the catabolic rate, or a relative decrease in the synthetic rate.

The rate of  $^{35}\text{S}$  incorporation by cultured explants of equine articular cartilage has been used commonly as a comparative measure of proteoglycan synthesis in *in vitro* trials. This method has also been used to infer what the *in vivo* synthetic rate may have been immediately prior to removal of the explant (Mankin *et al.*, 1972; Behrens *et al.*, 1976; Tyler, 1985; Chunekamrai *et al.*, 1989; Fubini *et al.*, 1993). However, the rate of  $^{35}\text{S}$  incorporation can also vary with the  $\text{SO}_4^{2-}$  concentration of the medium, the time each explant is exposed to the radiolabel, and the timing of the radiolabelling with respect to the start of the culture (Maroudas & Evans, 1974). Injection of  $\text{Na}_2^{35}\text{SO}_4$  and glycine- $\text{H}_3$  directly into the joints of live rabbits has also been used to compare relative proteoglycan and protein synthetic rates between joints (Behrens *et al.*, 1976; Mankin & Conger, 1966).

The Bitter & Muir (1962) assay for uronic acid has commonly been used to estimate the glycosaminoglycan content of articular cartilage (Glade *et al.*, 1983; Tyler, 1985; Trotter *et al.*, 1991). This assay estimates the chondroitin sulphate portion of the total glycosaminoglycan content of articular cartilage only. The relative proportions of chondroitin sulphate to keratansulphate vary with age, site, depth from the articular surface, and joint pathology.

Cationic dyes, such as alcian blue and dimethylmethylene blue (DMB), have been used to quantify released or extracted proteoglycans (Dingle *et al.*, 1979; McIlwraith & Van Sickle, 1981; Jubb, 1984; Tyler, 1985). Although these dyes bind to both major types of glycosaminoglycan present in articular cartilage, the resulting absorbance produced (525 nm) is much greater for chondroitin sulphate than for keratansulphate. Extraction techniques produce variable yields of proteoglycans. Furthermore, yield percentages tend to be affected by actual proteoglycan content (Stockwell, 1979). The DMB method has been increasingly used in comparison to other assays because of its relative simplicity, improved sensitivity, and ease of automation. When used in conjunction with tissue culture systems the DMB method can be subject to interference from several of the components of either the media or tissue digest (Sabiston *et al.*, 1985; Farndale *et al.*, 1986; Templeton, 1988). Proteolysis in combination with other purification techniques can remove much of this interference (Sabiston *et al.*, 1985; Farndale *et al.*,

1986). However, all reference standards should be spiked with similar concentrations of the interfering components, and be subjected to the same procedures also (Sabiston *et al.*, 1985). The use of shark chondroitin sulphate as a reference standard may result in an underestimation of the true amount of mammalian chondroitin sulphate present (Stockwell, 1979))

Histochemical staining techniques employing safranin-O and alcian blue have been used to assess variation in glycosaminoglycan content between histological sections of articular cartilage (Lust *et al.*, 1972; McIlwraith & Van Sickle, 1981; Hurtig *et al.*, 1988; Shamis *et al.*, 1989; Chunekamrai *et al.*, 1989). The number of negatively charged groups associated with a glycosaminoglycan molecule varies along its length and with its type (Wasteson & Lindahl, 1971). The affinity of these charged groups for some of the cationic dyes can vary with pH and competition from other cations. Quite apart from any trial differences, variations may also result from variable proteoglycan losses during processing of the articular cartilage samples prior to staining. As such, the resultant staining intensities and the distribution of staining intensities observed may not be representative of the initial proteoglycan content (Hunziker *et al.*, 1992).

Assays for both individual and total amino sugar content can be used to quantitatively estimate glycosaminoglycan content also (Mankin, 1974; Glade *et al.*, 1983; Richardson & Clark, 1990; Vachon *et al.*, 1991). Keratansulphate and chondroitin sulphate have equal proportions of amino sugar. Hyaluronic acid and glycoproteins also contribute to the total assayable amino sugar levels, but their contributions are considered small in comparison to those coming from keratansulphate and chondroitin sulphate. Analytical techniques used to estimate either total or individual amino sugar content include modifications of the Elson & Morgan (1933) colorimetric assay for amino sugars, gas liquid chromatography methods, and methods involving ion chromatography. These all require extensive hydrolysis of the proteoglycans to yield isolated amino sugar molecules before analysis, and hence give no indication of the distribution or size of the proteoglycans.

## 5. Response to injury

The loss of proteoglycans is recognised as an early response of articular cartilage to injury. The collagen structural framework is much more susceptible to mechanical damage if the proteoglycan content is depleted (McIlwraith & Van Sickle, 1981; Malesud *et al.*, 1987). Whether damage to the collagen structural framework precedes or follows proteoglycan depletion, both are considered inextricably linked in the progressive degeneration of articular cartilage in the horse (McIlwraith & Vachon, 1988).

It is not known whether the depletion of proteoglycans found in many cases of degenerative joint disease involves an increase in chondrocyte mediated catabolism, or whether the cartilage damage is the result of extrinsic enzymes originating from the synoviocytes, bacteria, or migrant leucocytes (Fell & Jubb, 1977; McIlwraith & Van Sickle, 1981; McIlwraith, 1982; Martel-Pelletier *et al.*, 1984; Hurtig, 1988; McIlwraith & Vachon, 1988; May *et al.*, 1991). Cytokine and drug induced suppression of synthesis may also be a significant factor in the chronic progression of some osteoarthritic conditions (Behrens *et al.*, 1974; Tyler, 1985; May *et al.*, 1992).

Chondrocytes have been shown to produce catabolic enzymes in response to a number of adverse conditions (Sapolsky *et al.*, 1981; May *et al.*, 1991). These include physical trauma, chemical irritants, pH changes, cytokines, inflammatory mediators or modulators, and inadequate supplies of nutrition. The cytokines and inflammatory mediators and modulators can originate from the chondrocytes themselves, synoviocytes, subchondral bone, granulation tissue, or leucocytes which have migrated into the synovium or synovial fluid (Dingle *et al.*, 1979; Morales, 1984; Tyler, 1985; Hickery *et al.*, 1990). Tumour necrosis factor, prostaglandin E<sub>2</sub> and interleukin-1 are thought to be involved in both stimulating increased enzymatic production, and in activating the intrinsic enzymatic degradation in articular cartilage (Dingle, 1984; May *et al.*, 1991; Price *et al.*, 1992; May *et al.*, 1992). Catalytic amounts of the neutral metalloproteinase stromelysin, once activated, can also activate at least one other pro-metalloproteinase found in articular cartilage (Ogata *et al.*, 1991).

Very little work has been done to investigate the types of enzymes involved in the catabolism of equine articular proteoglycans. As a consequence, much of the information concerning the mechanisms involved in equine articular cartilage catabolism must be extrapolated from work done on other species. However, increased amounts of the neutral metalloproteinase stromelysin have been shown to be produced by equine chondrocytes cultured under adverse conditions (May *et al.*, 1991).

Human articular cartilage contains a number of neutral and acid metalloproteinases capable of catabolizing proteoglycans. Much of the metalloproteinase content exists either in the latent forms or is inhibited by factors within the cartilage (Woessner & Selzer, 1984; Martel-Pelletier *et al.*, 1985; Flannery *et al.*, 1992). Two studies have reported higher active concentrations of neutral and acid metalloproteases in osteoarthritic cartilage, but in both cases the differences were not statistically significant (Martel-Pelletier *et al.*, 1984; Martel-Pelletier *et al.*, 1985). However, human osteoarthritic cartilage has been shown to contain both an elevated total neutral and acid metalloproteinase content (Sapolsky *et al.*, 1973; Sapolsky *et al.*, 1976; Martel-Pelletier *et al.*, 1985; Martel-Pelletier *et al.*, 1985).

Sachs *et al.* (1982) and Goldberg *et al.* (1984) described three types of changes in the metachromatic staining of human osteoarthritic cartilage. Type A is predominantly characterised by a lack of pericellular staining, giving rise to a halo-like appearance around the lacunae. Type B involves diminished staining in the interterritorial areas of the matrix, while Type C is characterised by a more irregular staining of the matrix. It has been postulated that metalloproteinases which have optimum effects at acid pH are primarily involved in intracellular and pericellular digestion (Pelletier *et al.*, 1987). In contrast, neutral metalloproteinases are more likely to also be involved in the degradation of territorial and interterritorial matrix proteoglycans (Sapolsky *et al.*, 1976; Bayliss & Ali, 1978; Martel-Pelletier *et al.*, 1984). However, at least one of the acid metalloproteinases able to be extracted from human articular cartilage, still retains 44% of its maximal activity at pH 7.5 (Woessner & Selzer, 1984; Azzo & Woessner, 1986).

Chondrocyte necrosis and hypocellularity may be associated with areas of mechanical damage, and hypertrophy and cluster formation have commonly been described around the periphery of damaged areas (McIlwraith & Van Sickle, 1981; Hurtig, 1988; Shamiš *et al.*, 1989).

Equine articular cartilage has a very limited capacity to mount a reparative response to injury (McIlwraith & Vachon, 1988). The type and effectiveness of repair are influenced by the size, thickness and anatomical location of the defect (Riddle, 1970; Convery, 1972; Grant, 1975; Vachon *et al.*, 1986; Hurtig, 1988; Shamiš *et al.*, 1989; Desjardins & Hurtig, 1990). Articular cartilage is avascular and thus can not mount a classical inflammatory response to injury. There are three mechanisms which can contribute to its repair (Sullins *et al.*, 1985; Hurtig, 1988; Desjardins & Hurtig, 1990):

- (1) **Intrinsic repair**, which relies upon the limited mitotic capability of chondrocytes and a short-lived increase in the biosynthetic rate of proteoglycans and collagen.
- (2) **Matrix flow**, which involves the migration of matrix components from the perimeter to the centre of a lesion.
- (3) **Extrinsic healing**, which involves cartilaginous metaplasia of granulation tissue originating from subchondral bone, and can occur only when the lesion penetrates the calcified zone.

Repair of damaged areas of the collagen network is relatively slow, and generally results in areas of articular cartilage which are structurally less ordered and functionally inferior (French *et al.*, 1989; Richardson & Clark, 1990). Proteoglycans are synthesised at a faster rate but require the structural integrity of the collagen framework if the original functional capability of the cartilage is to be restored. Reduced proteoglycan concentrations in tissue cultured cartilage explants have been associated with an increased rate of proteoglycan synthesis (Handley & Lowther, 1977; Sandy *et al.*, 1980; Sah *et al.*, 1988). However, the specific mechanisms regulating proteoglycan synthesis remain unknown (Tyler, 1985; Schneiderman *et al.*, 1986; Gray & Gottlieb, 1983).

## **B. USE OF INTRA-ARTICULAR CORTICOSTEROIDS IN THE HORSE**

Since their first reported use in the horse by Wheat (1955), corticosteroids have gained widespread use in the treatment of traumatic arthritis and the management of degenerative joint disease (McKay & Milne, 1976; Hackett, 1982; Trotter *et al.*, 1991). Clinically they reduce swelling and pain associated with inflammation of the soft tissues of the joint, resulting in improved function. They do not directly affect the initiating causes of inflammation, but non-specifically limit its full manifestation. The ability of corticosteroids to mask the clinical signs associated with joint damage while not affecting its cause, has led to much debate and a variety of international regulations concerning their use in competition horses. The New Zealand Racing Conference, New Zealand Harness Racing Conference, and New Zealand Equine Federation, all ban any corticosteroid administration which results in detectable urine or blood concentrations on competition days.

### **1. Anti-inflammatory mechanism of action**

Corticosteroids are thought to exert most of their biological activity through inducing, or inhibiting, the synthesis of new polypeptides via transcriptional regulation (Raz *et al.*, 1989; Raz *et al.*, 1990). Steroid molecules enter cells and bind to receptors, resulting in complexes capable of regulating the transcription of specific genes. Induced mRNA diffuses out of the nucleus, binds to the ribosomes and gives rise to new polypeptides. The anti-inflammatory effects of corticosteroids are mediated via four main mechanisms: (i) inhibition of arachidonic acid release, (ii) inhibition of inducible cyclo-oxygenase (COX-2) synthesis, (iii) inhibition of the synthesis and effects of cytokines, and (iv) inhibition of the generation of granulation tissue.

Corticosteroids are thought to inhibit the release of arachidonic acid from the lipid bilayer of cell membranes mainly by stimulating the synthesis and release of lipocortin (Flower & Blackwell, 1979). Lipocortin, a member of the annexin family of calcium binding proteins, acts by inhibiting free calcium ions activating membrane bound phospholipase A<sub>2</sub> from releasing arachidonic acid (Aarsman *et al.*, 1987; Davidson *et*

*al.*, 1987). Free arachidonic acid is a primary substrate for both the cyclo-oxygenase and lipoxygenase metabolic pathways, which ultimately produce the pro-inflammatory prostanooids and leucotrienes. Corticosteroids may also inhibit the synthesis and expression of phospholipase A<sub>2</sub> directly (Nakano *et al.*, 1990). However, doubt exists as to the clinical significance of these mechanisms of action, as phospholipase A<sub>2</sub> mediated release of arachidonic acid is not the only, or possibly even the most important, source of free arachidonic acid in inflamed tissues (Habenicht *et al.*, 1990; DeWitt, 1991).

The recent discovery of an isoenzyme of cyclo-oxygenase, variably named cyclo-oxygenase-2 (COX-2), inducible prostaglandin G/H synthase (PGHS-2) or glucocorticoid-regulated inflammatory prostaglandin G/H synthase (griPGHS), and whose production is affected by the corticosteroids, has significantly advanced the understanding of the anti-inflammatory effects of these drugs (Xie *et al.*, 1991; Kujubu *et al.*, 1991; O'Banion *et al.*, 1991). Concentrations of both PGHS-2 mRNA and enzyme have been shown to dramatically increase in response to growth factors, cytokines and oncogene activation. Both basal and induced concentrations of PGHS-2 mRNA can be suppressed by dexamethasone, and some evidence exists for both a transcriptional and post-transcriptional effect. In contrast PGHS-1 expression remains relatively constant.

Cytokines are defined as soluble peptides produced by one cell affecting the activity of other cell types (Price *et al.*, 1992). Corticosteroids can inhibit both the production and effects of many cytokines. Potential sources of cytokines in traumatised or inflamed joints include the chondrocytes themselves, synovial cells, and inflammatory cells which have migrated into the synovium (Ollivierre *et al.*, 1986). Several cytokines have been implicated in the pathogenesis of degenerative joint disease (Price *et al.*, 1992). Examples of cytokines which have catabolic or pro-inflammatory effects include, interleukin-1 (IL-1), the tumour necrosis factors (TNF- $\alpha$ , TNF- $\beta$ ), platelet activating factor (PAF), and plasminogen activator.

Produced by neutrophils, **plasminogen Activator** facilitates the migration of neutrophils to inflammatory sites by activating fibrinolysis and hydrolysis of proteins (Haynes, 1991; Price *et al.*, 1992). Furthermore, it may play an important role in activation of the metalloproteases involved in cartilage matrix catabolism (Campbell *et al.*, 1988).

**Platelet activating factor (PAF)** is produced by platelets, mast cells, monocytes, neutrophils, eosinophils, several types of renal cells, and vascular endothelium. It causes bronchoconstriction, vasodilation, and increases vascular permeability. Also, PAF promotes platelet and leucocyte aggregation, and is chemotactic for eosinophils, neutrophils, and monocytes. PAF is not stored by cells, but is synthesized, from cell membrane phospholipids, in response to adverse stimulation. Synthesis involves reactions catalysed by phospholipase A<sub>2</sub> and lyso-PAF acetyltransferase, both of which require free Ca<sup>2+</sup> ions to become active (Campbell, 1990). Corticosteroids can inhibit both the synthesis and effects of PAF. Inhibition of PAF synthesis is thought to be the result of corticosteroid mediated induction of the synthesis and release of the calcium binding protein lipocortin (Haynes, 1991).

**Interleukin-1 (IL-1)** is principally produced by monocyte macrophages but is also produced by most cell types under stress, including synoviocytes and chondrocytes (Ollivierre *et al.*, 1986). Raised synovial fluid concentrations of IL-1 have been found in human and equine patients suffering from osteoarthritis (Wood *et al.*, 1983; Morris *et al.*, 1990). Interleukin-1 stimulates the synthesis and activation of a range of inflammatory mediators and catabolic enzymes from a variety of different cell types, and inhibits the synthesis of new proteoglycan aggregates and collagen fibrils by chondrocytes (Morris *et al.*, 1990; MacDonald *et al.*, 1992). However, some debate exists over the clinical significance of the chondrocyte mediated catabolic effects of IL-1 at clinically relevant concentrations (Hickery *et al.*, 1990). Interleukin-1 also causes activation of T lymphocytes, stimulates fibroblast proliferation, and enhances hepatic synthesis of 'acute phase' proteins. It is chemotactic for leucocytes and causes neutrophilia by stimulating release of neutrophils into the vasculature (Price *et al.*, 1992). Corticosteroids are thought to reduce the synthesis of IL-1 by inhibiting

transcription of its mRNA. However, they can also inhibit the actions of IL-1 through a variety of other mechanisms (Haynes, 1991; Lane *et al.*, 1992).

**Tumour necrosis factor**, originally known as cachectin, has a similar range of biological effects to IL-1. Mainly produced by monocyte macrophages and lymphocytes, TNF stimulates bone and cartilage resorption through the induction of prostaglandins and metalloproteinases (Saklavalala, 1986; Brunning & Russell, 1989). The effects of TNF and IL-1 are additive but are not mediated by the same receptor (Bird & Saklavalala, 1986). TNF also mediates some of the cytotoxic effects of activated T lymphocytes, and high plasma concentrations of TNF have been implicated in the pathogenesis of septic shock. Both the synthesis and some of the effects of TNF can be inhibited by corticosteroids.

Corticosteroids can also inhibit the synthesis and/or effects of the class of cytokines more commonly known as the **growth factors**. These include the insulin-like growth factors, transforming growth factors, platelet derived growth factor, and fibroblast growth factor (Price *et al.*, 1992). Insulin-like growth factor-1 can decrease degradation and promote synthesis of proteoglycan in cartilage exposed to either IL-1 or TNF (McQuillan *et al.*, 1986; Tyler, 1989). The effects of transforming growth factor  $\beta$  on the activity of chondrocytes are less clear, but it may play a role in the elevated proteoglycan synthesis and cell proliferation in osteoarthritic cartilage (van der Morales & Roberts, 1988; van der Kraan *et al.*, 1992). Platelet derived growth factor, fibroblast growth factor, and several others are involved in the complex series of events resulting in fibroblast proliferation and the generation of granulation tissue (Shoemaker *et al.*, 1992). The ratio of anticatabolic to anabolic effects in different clinical situations is the subject of much debate and is one of the major points of contention concerning their use.

The exact mechanism of action by which steroids inhibit cell division and even cause cell death, is not known (Chunekamrai *et al.*, 1989). However, inhibition of normal metabolism is likely to be an important mechanism of action (Glade *et al.*, 1983). High concentrations of steroids have been associated with disruption of the normal structure

of intracellular organelles (Behrens *et al.*, 1975; Ohira & Ishikawa, 1986). Cytotoxic effects have been shown to be both dose and time dependant (Hainque *et al.*, 1987). Certain cell types, for instance lymphocytes, have an inherent increased sensitivity to these effects (Tobin, 1979; Haynes, 1991).

Most of the research into the mechanisms of action of corticosteroids has been done in species other than the horse. However, two equine reports have specifically investigated what effects clinically recommended doses of betamethasone have on the PGE<sub>2</sub>-like activity and leucocyte numbers of carageenan induced acute inflammatory exudate. Single betamethasone doses (80 µg kg<sup>-1</sup>, IV), administered several hours prior to the carageen, had no significant effect on the PGE<sub>2</sub>-like activity of the subsequently produced exudate. Furthermore leucocyte numbers in the exudate were unexpectedly and paradoxically increased in the exudate, both with single dosing and with multiple dosing for four days before inducing the localised inflammatory reaction. PGE<sub>2</sub>-like activity was reduced only after the horses had received either multiple 80 µg kg<sup>-1</sup> doses or a very large single dose (1 mg kg<sup>-1</sup>). The authors concluded that the data failed to support the hypothesis that the actions of betamethasone in the horse are wholly attributable to the inhibition of phospholipase A<sub>2</sub> and a consequent suppression of eicosanoid production.

## **2. Manufacturer recommendations**

There are a number of soluble and depot corticosteroid formulations registered in New Zealand for intra-articular administration. Recommended dose ranges are wide and are predominantly based on the volume of synovial fluid in the joint being treated. Both methylprednisolone acetate products available recommend intra-articular doses of 40-240 mg. Neither product specifically stipulates whether this dose is a per joint or a per horse dose, although the product sheet of Depo-Medrol® suggests that 120 mg is the average dose for a large joint.

### 3. Pharmacokinetics

Intra-articular (I/A) injections of methylprednisolone acetate (MPA) are essentially placed at or close to their site of action. The rapidity, intensity, and duration of the effects are thus primarily affected by the rates of (a) disintegration of the particles, (b) dissolution of MPA, and (c) hydrolysis of MPA to methylprednisolone (MP), the active form of MPA, and not by systemic distribution, metabolism, and elimination characteristics.

Post-mortem inspections of joint capsules have revealed visible MPA deposits for at least three days post-injection (Autefage *et al.*, 1986), and up to 13 days following a single I/A administration (Pool *et al.*, 1981). Chunekamrai *et al.* (1989) reported that after five weekly I/A injections, crystals of MPA were seen in most of the synovial fluid aspirates immediately prior to the subsequent weekly administrations. These observations contrast sharply with the often quoted reports of Wilson *et al.* (1953) and Zacco *et al.* (1954) which concluded that within 2 hours of I/A administration, most of the hydrocortisone acetate dose was contained within the synoviocytes or cells in the synovial fluid, and any remaining drug was almost totally hydrolysed.

Doses of intra-articular depot corticosteroids have traditionally reflected arbitrary and empirical volume based dosage schedules. The aim of these has been the maximal suppression of pain and inflammation within a joint for as long as possible, with dosage intervals determined primarily by pharmacological effect (Autefage *et al.*, 1986; Trotter *et al.*, 1991). Only one study has reported the equine synovial fluid kinetics of MPA and MP following I/A administration of a MPA formulation (Autefage *et al.*, 1986). A total dose of 111.2 mg of MPA<sup>1</sup>, corresponding to 100 mg of MP, was injected into the right tibiotarsal joint of five adult horses. Even though MPA is practically insoluble in water, high synovial fluid concentrations ( $289 \pm 284 \mu\text{g ml}^{-1}$ ) of MPA were recorded at the first sampling time (2 hours post-injection), and MP concentrations were maximal ( $58.9\text{-}379.5 \mu\text{g ml}^{-1}$ ) at either the first or second sampling time (2-10 hours after

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<sup>1</sup> Depo-Medrol, 40 mg ml<sup>-1</sup>, Upjohn.

administration). These results indicated that MPA is relatively rapidly hydrolysed to its active form, presumably by esterases present in the synovial fluid, and should be considered a rapidly acting drug when administered by the I/A route in the horse (Autefage *et al.*, 1986). The disposition kinetics of MPA were best described by a biexponential equation of the form: Synovial concentration =  $Ae^{-\alpha t} + Be^{-\beta t}$ , with the half-time of the second phase estimated to be  $11.81 \pm 7.37$  hours.

Even though MPA was present in detectable concentrations (10-20 ng mL<sup>-1</sup> of synovial fluid) for only 3-6 days in this study, MP was present in pharmacologically significant concentrations (37 ng mL<sup>-1</sup> or greater) from 4.8 to more than 39 days. The disposition kinetics of MP were also best described by a biexponential equation. After reaching maximal values at either the first or second sampling time, synovial fluid concentrations of MP fell progressively for the first five days with a half-time of 9.95 hours, then decreased more slowly with an apparent half-time of 115 hours (Autefage *et al.*, 1986). However, very large differences were observed between horses with the half-time of the second phase varying from 37-208 hours; the authors concluded that these differences were possibly due to variable disintegration of the deposits of MPA in different locations, and that this might explain the drugs variably sustained action in different joints. They further concluded that the duration of detectable concentrations of MP (10 to 20 ng mL<sup>-1</sup>), following I/A administration of MPA in a horse, was largely unpredictable. Thus no pharmacokinetically based recommendations concerning the inter-dose interval could be offered.

MPA was not detected in plasma (sensitivity 2-3 ng mL<sup>-1</sup>) at any time after I/A administration (Autefage *et al.*, 1986). However, low concentrations of MP (less than 5 ng mL<sup>-1</sup>) were detected in all horses for about 24 hours following I/A administration of MPA. The disposition kinetics of intravenously administered MP have best been described by a triexponential equation of the form: Plasma concentration =  $Pe^{-\pi t} + Ae^{-\alpha t} + Be^{-\beta t}$  (Autefage *et al.*, 1986). The half-life calculated from the slope of the terminal portion of this curve was  $172 \pm 88$  minutes (range of values 93-308.5 minutes).

Systemic distribution, metabolism, and elimination characteristics were thus considered not to greatly affect synovial fluid concentrations of either MPA or MP in the horse following I/A administration of MPA.

Although MPA is predominantly administered I/A to ameliorate the effects of joint pain and inflammation, the effect of joint inflammation on the synovial fluid kinetics of I/A administered MPA in horses has not been studied.

#### **4. Clinical use**

Corticosteroids can be beneficial in the relief of pain and the treatment or management of a wide range of intra- and peri-articular conditions. Use of the intra-articular route of administration allows high concentrations of corticosteroid to be achieved locally in the synovium and joint capsule, while high plasma concentrations and their consequent systemic effects are avoided (Autefage *et al.*, 1986).

Therapeutically, methylprednisolone acetate can be used to inhibit both the exudative and cellular manifestations of acute inflammation. It can limit the potentially damaging effects of the inflammatory process on joint tissues, while a more specific therapy addresses the primary cause of the inflammation (May *et al.*, 1987). Through inhibiting the exudative and the cellular responses to injury, corticosteroids help reduce chronic joint thickening and scar formation and maintain an effective circulation and nutrient supply to articular cartilage and surrounding tissues (McIlwraith & Vachon, 1988).

Intra-articular corticosteroids may break the seemingly self-perpetuating cycle of inflammation initiated by some joint injuries. The permanence of this effect after the drug has been eliminated, and the effect of corticosteroids on the progression of chronic inflammation, are less clear (May *et al.*, 1987). Owen (1980) concluded that I/A administration of depot corticosteroids may be beneficial if lesions are confined to the soft tissues of the joint, are not associated with joint laxity, and if the joint is rested for an appropriate period following therapy.

The analgesic effects of I/A corticosteroids arise as a result of their anti-inflammatory actions (Higgins & Lees, 1984). Corticosteroids inhibit the production of several inflammatory mediators capable of stimulating or hypersensitizing peripheral nerve endings. Also, reduced inflammation relieves pressure on capsular insertions (May *et al.*, 1987). The combined anti-inflammatory and associated analgesic actions can result in the return of function to an inflamed joint irrespective of its initiating cause. Consequently, intra-articular depot corticosteroids can be used specifically to mask the clinical signs of joint inflammation, so as to facilitate the continued training or competing of an otherwise unsound horse (McKay & Milne, 1976; Hackett, 1982).

## 5. Adverse effects on joints

Despite well documented cases of intra-articular corticosteroids being implicated in the pathogenesis of progressive joint degeneration, no pathognomonic lesion for so-called 'steroid induced arthropathy' has been described in the horse (Pool *et al.*, 1981; McIlwraith, 1989). There is general agreement that most of the steroid arthropathies result from the intra-articular injection of corticosteroids into joints that either already have significant cartilage or bone disease, or are not properly rested during and after therapy (McKay & Milne, 1976; Pool *et al.*, 1981; McIlwraith & Vachon, 1990).

Use of corticosteroids in structurally damaged joints, to facilitate continued training and competition, exposes the compromised joint to a greater risk of further damage (Pool *et al.*, 1981; Trotter *et al.*, 1991). Corticosteroids inhibit tissue repair and normal turnover of fibrous tissue (Shoemaker *et al.*, 1992). Intra-articular administration of depot corticosteroids when chip fractures are present can compromise healing of the fracture and lead to more extensive and severe joint degeneration, especially if combined with continued training (Meagher, 1970). Pre- or post-surgical use can interfere with normal wound healing and may predispose to post-surgical infection (Meagher, 1970). Intra-articular administration of depot corticosteroids in cases of infectious arthritis compromises the defensive response at the site of infection, and can ultimately lead to increased agent propagation, dissemination and joint destruction

(Tulamo *et al.*, 1989). Low doses of corticosteroids given over several months interfere with normal development of joints in foals (Glade *et al.*, 1983).

Post injection 'flare' is a rare but well recognised possible sequel to intra-articular depot corticosteroid administration (Owen, 1980; Hackett, 1982), as are cases of laminitis (Vernimb *et al.*, 1977; McIlwraith, 1987). However, the doses normally administered, and the rate of systemic absorption, are such that systemic immune responses and adrenal responsiveness are not usually affected (Autefage *et al.*, 1986).

Intra-articular use of corticosteroids remains controversial, but widespread, in the racing industry. There is little reliable information on the risk/benefit ratio associated with their use (Pool *et al.*, 1981). However, the following recommendations have been proposed for the use of intra-articular depot corticosteroid therapy in the horse (Gabel, 1978; McIlwraith, 1987):

- (1) They may be indicated for the treatment of synovitis and capsulitis and are very effective in this regard.
- (2) Aseptic technique is mandatory.
- (3) Steroids are contraindicated if the joint is not radiographed for structural damage, or if there is instability in the joint.
- (4) The response is less satisfactory where degenerative joint disease is present.
- (5) Surgery should be approached cautiously where there is a history of prior corticosteroid administration in the horse (this is of less serious concern when surgery is performed arthroscopically)
- (6) It should always be remembered that corticosteroids tend to slow the rate of healing of articular cartilage, joint capsule, ligaments, and bone, and so additional forced exercise may compound the injuries and damage that have already occurred

## C. EFFECTS OF CORTICOSTEROIDS ON ARTICULAR CARTILAGE

A variety of species, treatment regimes, and analytical techniques have been used to investigate the effects of corticosteroids on articular cartilage. Despite much research, debate, and widespread use in equine practice, there is little objective data on their potential effects on equine articular cartilage at the concentrations used clinically (Trotter *et al.*, 1991).

The response of articular cartilage to experimental conditions differs from species to species. For instance, the half-life of the proteoglycans in rabbit stifle articular cartilage has been estimated to be as short as 10 days (Mankin & Lippiello, 1969), while in man the half-life of proteoglycans is estimated to be 300-800 days, depending on anatomical site (Maroudas, 1975). There is also wide variation in the magnitude and type of forces imposed on the articular cartilages of different joints and different species. Corticosteroids have a variety of metabolic effects on different tissues and many of these vary with both the type of corticosteroid and its concentration. Thus, for a given research result, it is important to recognise the joint type, species, corticosteroid involved, dose rate, and experimental conditions prevailing. However, information can still be gleaned from the variety of animal models and techniques used to gain an insight into what is likely to occur in equine joints.

### 1. Normal articular cartilage

One of the primary rationales for the therapeutic use of I/A corticosteroids is to protect articular cartilage from damage caused by the products of inflammation. Consequently it is important to know what effects corticosteroids may have on normal articular cartilage, and to relate these effects to the size, number, and frequency of administration.

(i) **Chondrocyte morphology**

Repeated I/A administration of large doses of corticosteroid into the stifle joints of rabbits have resulted in an increased incidence of histologically observable degenerative changes in treated joints when compared to the 'untreated' contralateral joints (Mankin & Conger, 1966; Salter *et al.*, 1967; Kopta & Blosser, 1969; Behrens *et al.*, 1975; Ohira & Ishikawa, 1986). Degenerative changes included chondronecrosis, intra-cartilaginous cysts, fibrillation and fissure development. Ultrastructurally, atrophy of the golgi apparatus, disruption of the endoplasmic reticulum, and deposition of hydroxyapatite have also been observed (Behrens *et al.*, 1975; Ohira *et al.*, 1986).

Several studies have investigated histological changes in equine carpal articular cartilage following repeated I/A administration of a methylprednisolone acetate formulation<sup>2</sup> into clinically normal joints. Two studies reported degenerative cellular changes including empty lacunae, hypocellularity and intra-cartilaginous cysts (Chunekamrai *et al.*, 1989; Shoemaker *et al.*, 1992), and another reported 'early osteoarthritic lesions' but no cyst-like degeneration (Marcoux, 1977). In contrast, Trotter *et al.* (1991) found no increase in the incidence of degenerative lesions in any of the methylprednisolone treated joints. Repeated systemic corticosteroid administration in foals has also resulted in degenerative cellular changes (Glade *et al.*, 1983). Generally, the experiments resulting in a higher incidence of degenerative chondrocytes involved frequent I/A administrations of high doses of corticosteroids (Chunekamrai *et al.*, 1989; Shoemaker *et al.*, 1992). Lower doses of methylprednisolone acetate and/or longer dose interval showed fewer or no changes in the chondrocytes (Marcoux, 1977; Trotter *et al.*, 1991).

(ii) **Matrix biosynthesis and depletion; *in vivo* results**

Mankin & Conger (1966), using radiolabelled glycine, concluded that slightly soluble hydrocortisone acetate injected intra-articularly profoundly reduces the rate of protein synthesis in rabbit stifle articular cartilage. The duration of this effect was clearly dose

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<sup>2</sup> Depo-Medrol, 40 mg ml<sup>-1</sup> methylprednisolone acetate, Upjohn Inter-American Corp.

dependant. When 0.25 mg was administered the metabolic rate recovered in only 3 days and the opposite joint was not identifiably affected. When 2.5 mg was used recovery of protein synthesis took an average of 14 days and the opposite joint was also initially affected. Behrens *et al.* (1975) investigated the effects of 2-12 weekly I/A administrations of 25 mg hydrocortisone acetate per knee on rabbit stifle articular cartilage. Average proteoglycan content, as shown by hexosamine assay, diminished as the number of injections increased. Also, differences in radiolabel incorporation between treated and untreated joints demonstrated there were persistent and highly significant reductions in protein, collagen, and proteoglycan synthesis. Mankin *et al.* (1972) reported similar effects in rabbit stifle articular cartilage following daily intramuscular administration of 4.5 mg kg<sup>-1</sup> cortisone acetate for up to 9 weeks.

To date, only two studies have compared the biosynthetic rates of cartilage from normal and methylprednisolone treated equine joints (Chunekamrai *et al.*, 1989; Fubini *et al.*, 1993). For both investigations, articular cartilage was harvested from a small number of horses and cultured in media supplemented with 10  $\mu$ Ci of Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> ml<sup>-1</sup>. Counts per minute (cpm)  $\mu$ g<sup>-1</sup> glycosaminoglycan, and cpm mg<sup>-1</sup> (wet weight) of cartilage were used as relative measures of the rate of proteoglycan synthesis occurring within the explants. Fubini *et al.* (1993) reported no significant difference in the rate of proteoglycan synthesis between explants from control joints versus those from joints which had had a single I/A administration of a methylprednisolone acetate formulation 3 weeks previously. In contrast, Chunekamrai *et al.* (1989) reported the rate of proteoglycan synthesis of cartilage explants from treated joints was reduced to 17.04% of control values 1 week after 8 weekly 120 mg joint<sup>-1</sup> I/A administrations of methylprednisolone acetate. Three weeks later the rate of synthesis had increased to 55.31% of control values, and by 8 weeks had further recovered to be 71.28% of control values. A concurrent experiment using a different joint of the same horses found that proteoglycan synthesis 16 weeks after a single I/A methylprednisolone acetate administration was increased, but the difference was not statistically significant (170.48% of the controls). The horses used in the experiments conducted by Chunekamrai *et al.* (1989) were subjected to many large doses of a methylprednisolone acetate formulation into multiple joints at short intervals. It was likely that

methylprednisolone acetate accumulated in the joints which had multiple administrations. It was also possible that sufficient methylprednisolone was absorbed to have exerted some systemically mediated effects.

Several studies have histochemically compared cartilage proteoglycan content from methylprednisolone acetate treated and untreated equine joints. Four of these studies reported a marked diminution of Safranin-O staining intensity in the articular cartilage of the methylprednisolone acetate treated joint (Pool *et al.*, 1981; Chunekamrai *et al.*, 1989; Trotter *et al.*, 1991; Shoemaker *et al.*, 1992). In contrast Marcoux (1977) could identify no loss of proteoglycan content in treated joints.

Only Chunekamrai *et al.* (1989) and Trotter *et al.* (1991) have attempted to quantitatively assay the cartilage proteoglycan content of treated versus untreated joints. Chunekamrai *et al.* (1989) used the dimethylmethylene blue assay of Farndale *et al.* (1982) as a relative measure of the sulphated glycosaminoglycan component of the cartilage proteoglycan content. One week after eight weekly administrations of 120 mg methylprednisolone acetate per joint, the relative proteoglycan content ( $\mu\text{g mg}^{-1}$  of dry weight) was about half that of the untreated joints. Trotter *et al.* (1991) used Bitter & Muir's (1962) uronic acid assay as a measure of the chondroitin sulphate component of the cartilage proteoglycan content and reported that the mean articular cartilage uronic acid content ( $\mu\text{g mg}^{-1}$  of dry weight) of the methylprednisolone acetate injected joints was only about 17% of that of the lactated Ringer-injected joints.

### (iii) Matrix biosynthesis and depletion; *in vitro* results

Jacoby (1976) cultured human articular cartilage explants from several joints for 8 days in two concentrations of hydrocortisone succinate (0.01 and 0.1  $\mu\text{g mL}^{-1}$ ) and hydrocortisone acetate (1000  $\mu\text{g mL}^{-1}$ ), and found no difference between treatment groups and controls in the amount of proteoglycan shed into the media. He concluded that hydrocortisone does not have a catabolic effect on the proteoglycan content of tissue cultured human articular cartilage. Proteoglycan content was biochemically estimated using assays for both uronic acid (Bitter & Muir, 1962) and hexosamine

content (gas liquid chromatography method). Tyler *et al.* (1982) found that the usual loss of proteoglycans associated with the culture of porcine cartilage explants was partially suppressed by the addition of  $1.0 \mu\text{g mL}^{-1}$  hydrocortisone succinate to the culture medium. Tyler *et al.* (1982) also showed that in the presence of  $1.0 \mu\text{g}$  hydrocortisone acetate  $\text{mL}^{-1}$ , the explants had less outgrowth of tissue from the cut surfaces than the controls.

Pool *et al.* (1981) cultured explants of equine distal femoral articular cartilage. There was a marked loss of Safranin-O staining after 3 days, and a complete loss after 7 days from explants cultured in the presence of 'low therapeutic concentrations of methylprednisolone', as compared with both the cultured and uncultured controls. However, this experiment was reported as part of a more general report and did not include many details.

## 2. Articular cartilage of arthritic joints

High concentrations of glycosaminoglycans have been found in the synovial fluid of horses with osteochondritis, traumatic arthritis and osteoarthritis (Alwan *et al.*, 1991). Increased concentrations of proteoglycans have also been found in the synovial fluid of human patients with acute inflammatory arthropathies (Saxne, *et al.*, 1987; Ratcliffe *et al.*, 1988). Locally injected corticosteroids induced a reduction in the proteoglycan content of the synovial fluid of human patients with a variety of osteoarthritic conditions (Saxne *et al.*, 1986). However, this study did not differentiate whether the reduction in synovial fluid proteoglycan content was due to a reduced synthesis or decreased catabolism.

Total neutral metalloproteinase activity in cartilage from rheumatoid arthritis patients was roughly eight times that of control subjects (Martel-Pelletier *et al.*, 1985). Concentrations of the active forms of the neutral metalloproteinases were also higher in rheumatoid arthritic cartilage than in normal cartilage, but were not statistically different. However, when the rheumatoid arthritis patients were split into treatment groups, those treated with corticosteroids had significantly lower concentrations of the

active forms of the enzymes than those not receiving corticosteroids. Furthermore, concentrations of the active forms of the enzymes present in the corticosteroid treated group were not significantly different from those of the normal cartilage controls. The authors concluded that their data strongly supported the theory that neutral metalloproteinase be involved in the destruction of rheumatoid arthritic cartilage and indicated corticosteroids can suppress the synthesis and/or activation of neutral metalloproteinases (Martel-Pelletier *et al.*, 1985).

In patients with primary osteoarthritis the total acid metalloproteinase activity of cartilage adjacent to osteoarthritic lesions was greater than cartilage from age matched clinically normal controls (Pelletier *et al.*, 1987). The active form of this enzyme was also elevated at these sites, but the difference was not statistically significant. Patients treated with corticosteroids had much lower enzyme concentrations than those on other treatments, but concentrations were not significantly different from those in normal cartilage. The authors concluded that corticosteroids may regulate the production and/or activation of all metalloproteinases in cartilage. It was not known whether the effects of corticosteroids were mediated by a suppression of substances from the synovium or through a direct effect on the chondrocytes (Pelletier *et al.*, 1987).

Equine chondrocytes subjected to adverse culture conditions produce increased concentrations of the neutral metalloproteinase stromelysin (May *et al.*, 1991), and joints with osteoarthritis have higher synovial fluid glycosaminoglycan concentrations than do clinically normal joints (Alwan *et al.*, 1991). However, reports are lacking on the specific effects of corticosteroids on osteoarthritic equine cartilage.

### **3. Articular cartilage repair**

Lesions which penetrate the calcified zone of articular cartilage heal predominantly by cartilaginous metaplasia of granulation tissue originating from the mesenchymal cells of the subchondral bone. Corticosteroids have a suppressive effect on both the proliferation and differentiation of granulation tissue responsible for this extrinsic form of healing (Meagher, 1970; Shoemaker *et al.*, 1992). Articular cartilage lesions which

do not breach the junction between the non-mineralised and mineralised cartilage heal only by intrinsic and matrix flow mechanisms. These repair processes have very limited potential for repair of substantial structural damage, and may also be inhibited by corticosteroids (Tyler *et al.*, 1982; Shamis *et al.*, 1989; Shoemaker *et al.*, 1992). Shoemaker *et al.* (1992) compared the effects of four weekly I/A administrations of 100 mg methylprednisolone acetate on (a) normal equine articular cartilage and (b) the healing of surgically induced osteochondral defects. There were no gross abnormalities in the articular cartilage adjacent to the induced lesions in the methylprednisolone treated joints. However, there was a higher incidence of chondrocyte morphological abnormalities in other areas of the treated joints compared with the untreated contralateral joints.

The pathogenesis of the cartilage degeneration clinically observed in many methylprednisolone treated joints remains unexplained. Furthermore, little is known of the possible dose relationship of the effects of methylprednisolone acetate on chondrocytes, yet in most other tissues the effects of corticosteroids are very dose dependant (Haynes, 1991).

## D. USE OF PHENYLBUTAZONE IN THE HORSE

Phenylbutazone is a non-steroidal anti-inflammatory drug (NSAID) which has been very commonly used in horses for over 30 years. Its anti-inflammatory and analgesic actions are used in the management of a variety of soft tissue and musculoskeletal inflammatory conditions (Lees & Higgins, 1985; Tobin *et al.*, 1986).

### 1. Molecular mechanism of action

The clinical effects of phenylbutazone result predominantly from inhibition of the cyclo-oxygenase pathway. Following insult, this pathway converts free arachidonic acid into thromboxane (TxA), prostacycline (PGI<sub>2</sub>), and the prostaglandins (Vane, 1976; Lees & Higgins, 1985). Phenylbutazone may also scavenge or interfere with the production of free radicals at sites of inflammation (Auer *et al.*, 1990). In addition to being potent mediators of inflammation, prostacycline and the prostaglandins also potentiate several other important pro-inflammatory mediators and catabolic enzymes (Higgins & Lees, 1984).

The specific enzyme to which phenylbutazone binds in the cyclo-oxygenase pathway, and the nature of this interaction, is the subject of some debate (Tobin *et al.*, 1986). Phenylbutazone is commonly reported to act via its 'inhibition of cyclo-oxygenase' (Vane, 1976; Lees *et al.*, 1986; Hardee & Moore, 1986). However, some ambiguity exists in the terminology, as 'cyclo-oxygenase' can be used to describe both the general metabolic pathway and the first enzyme of that pathway. The cyclo-oxygenase enzyme, variably named prostaglandin G/H synthase (PGHS) and prostaglandin endoperoxide synthase, actually has two activities: (a) A cyclo-oxygenase activity, which catalyses a bis-oxygenation of arachidonate to yield prostaglandin G<sub>2</sub> (PGG<sub>2</sub>), and (b) a peroxidase activity, which catalyses a two-electron reduction of the 15-hydroperoxyl group of PGG<sub>2</sub> to produce prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) (Smith *et al.*, 1992).

Some researchers have proposed that phenylbutazone may inactivate other enzymes in the cyclo-oxygenase pathway. Flower (1974) proposed that phenylbutazone antagonises the action of endoperoxidase isomerase, thus specifically blocking the formation of

prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). Reed *et al.* (1985) suggested that phenylbutazone may directly inhibit the action of both PGHS and prostacyclin synthase, with inactivation of the latter probably the more clinically important.

The recent discovery of an inducible isoenzyme of PGHS, PGHS-2, which has different NSAID affinities, may shed new light on the subject (Xie *et al.*, 1992). In the horse, inhibition of PGI<sub>2</sub>, PGE<sub>2</sub>, and TxA by phenylbutazone is firm evidence that at least in this species the mechanism(s) of action involves inactivation of one of the PGHS isoenzymes (Higgins *et al.*, 1984; Hardee & Moore, 1986; Lees *et al.*, 1986).

There are also various opinions on whether inhibition of the cyclo-oxygenase pathway by phenylbutazone is reversible or irreversible (Ku & Wasvery, 1973; Flower, 1974; Lees *et al.*, 1987). The restoration of thromboxane synthesis by equine platelets, which are anuclear and thus incapable of synthesising new enzymes, suggests that at least in this tissue phenylbutazone binds reversibly to PGHS (Kopp *et al.*, 1985; Hardee & Moore, 1986; Lees *et al.*, 1987).

The actions of NSAIDs can be divided into central and peripheral, central actions being analgesic and antipyretic, and the peripheral being anti-inflammatory, local analgesic, anti-endotoxaemic, and antithrombotic. In contrast to the significant central analgesic effects of some NSAIDs, the pain relief associated with phenylbutazone use is thought to result predominantly from its local anti-inflammatory effects. Phenylbutazone has no direct effect on pain perception in normal tissues. Its analgesic effects are mainly due to its inhibition of the synthesis of inflammatory mediators such as PGE<sub>2</sub> and PGI<sub>2</sub>, which normally hypersensitize the nerve endings surrounding injured tissue (Moncada & Vane, 1979; Kamerling, *et al.*, 1983).

Prostaglandin production in acute inflammation is thought to be initially regulated by the rate of release of arachidonic acid from cell membranes by activated phospholipases. However, as cyclo-oxygenase is physiologically inactivated during catalysis (DeWitt, 1991), and pharmacologically by a number of drugs (Smith *et al.*, 1992), both the initial concentration of cyclo-oxygenase and the rate at which it can be resynthesized soon

become the prime determinants of the rate at which arachidonic acid is metabolised to form the prostanoids (DeWitt, 1991).

Small differences in the structure of cyclo-oxygenase pathway enzymes may result in phenylbutazone having different affinities for the enzymes of the cyclo-oxygenase pathways of different tissues and different species (Lees & Higgins, 1985; DeWitt, 1991; Simmons *et al.*, 1993). Furthermore, the rate of synthesis of PGHS in different tissues can vary by several thousand percent (DeWitt, 1991). Minimum therapeutic plasma concentrations of phenylbutazone in man have been estimated to be 50-150  $\mu\text{g ml}^{-1}$  (Aarbakke, 1978). Much lower plasma concentrations (5-15  $\mu\text{g ml}^{-1}$ ) are associated with clinical efficacy in the horse (Jenny *et al.*, 1979; Gerring *et al.*, 1981). However, plasma concentrations are not a good measure of the duration of clinical effect, which normally exceeds pharmacokinetically derived predictions (Lees *et al.*, 1986).

The products of the cyclo-oxygenase pathway have physiological as well as pathophysiological functions. Many of the adverse effects of phenylbutazone are thought to be due to its interference in the normal physiological production of the prostaglandins and prostacycline, both of which are involved in the maintenance of the microcirculation of tissues (Whittle & Vane, 1984; Collins & Tyler, 1985). However, it was concluded in a recently reported equine toxicological study that phenylbutazone-induced gastric erosions in horses are primarily due to a direct toxic effect of the drug on the endothelium of the microvasculature and intestinal mucosa, and that the development of erosions is not mediated entirely by a reduction in normal physiological prostaglandin production (Meschter *et al.*, 1990). The results from this study are in agreement with those of several other investigations in different species (Whittle, 1981; Rainsford & Willis, 1982). The adverse effects of phenylbutazone are more severe in situations of compromised circulation and dehydration (Read, 1983; Gunsen & Soma, 1983; Behm & Berg, 1987), or when given at high dose rates for extended periods of time (Snow *et al.*, 1981; MacAllister, 1983; Traub *et al.*, 1983; Mackay *et al.*, 1983).

Phenylbutazone can also inhibit other enzymes, particularly through its ability to uncouple oxidative phosphorylation, and at relatively high concentrations suppresses proteoglycan synthesis (Whitehouse & Haslam, 1962; Whitehouse, 1964; Bostrom *et al.*,

1964; Domenjoz, 1966). However, it is not known if phenylbutazone inhibits proteoglycan synthesis, or other metabolic processes, in equine chondrocytes at the concentrations achieved clinically in synovial fluid.

## 2. Manufacturer recommendations

Recommendations concerning phenylbutazone dose, dosage interval, and treatment period vary widely with both product and route of administration. Recommended intravenous dose rates vary from 2.2-4.4 mg kg<sup>-1</sup> day<sup>-1</sup> for up to 5 days<sup>3</sup> to 5.8-11.6 mg kg<sup>-1</sup> day<sup>-1</sup> for 3 to 5 days<sup>4</sup> (both doses rates assuming a 450 kg horse). The latter dose rate applies to phenylbutazone in a phenylbutazone/isopyrin combination product, in which with isopyrin, in which phenylbutazone is reported to have a longer plasma elimination half-life than when administered alone (Jenny *et al.*, 1979).

Oral dose rates usually incorporate an initial loading dose of up to 8.8 mg kg<sup>-1</sup>, given in two divided doses on the first day, then progressively lower doses. One product has a recommendation that the treatment should be reassessed after 10 days<sup>5</sup>. Another product<sup>6</sup> recommends that no more than 4.4 mg kg<sup>-1</sup> should be administered daily for a maximum of 7 days. The other products have no recommendations concerning duration of treatment. The chronic nature of many of the ailments for which phenylbutazone is used means it is often administered for extended periods.

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<sup>3</sup> Butalone, Techvet laboratories limited.

<sup>4</sup> Isoprin, Techvet laboratories limited.

<sup>5</sup> Bute (paste), Techvet New Zealand limited.

<sup>6</sup> Myoton granules, Pitman-Moore.

### 3. Pharmacokinetics

Phenylbutazone (4-butyl-1, 2-diphenyl-3, 5-pyrazolidinedione) is an enolic acid with lipophilic properties, a pKa of 4.5 and a molecular weight of 308.37 Da. It has low aqueous solubility, and consequently most injectable preparations are alkaline solutions of the sodium salt.

#### (i) Absorption

Intravenously and orally administered formulations are the most commonly used phenylbutazone preparations in equine medicine. Bioavailability and the rate of absorption of orally administered phenylbutazone vary widely between horses and are influenced by the formulation of the drug and the type of ingesta present (Moss, 1972; Gerring *et al.*, 1981; Rose *et al.*, 1982; Sullivan *et al.*, 1982; Snow *et al.*, 1983; Bogan *et al.*, 1984; Maitho *et al.*, 1985; Soma *et al.*, 1985; Lees *et al.*, 1986; Lees *et al.*, 1988). Breed and age differences may also exist, and some products have the recommendation that lower initial dose rates should be administered to ponies and immature animals (Lees *et al.*, 1985).

#### (ii) Distribution

##### Plasma kinetics

The plasma disposition of phenylbutazone following intravenous injection can be described by a two-compartment open model. Phenylbutazone is 96-98.4% protein bound in equine plasma (Snow, 1981; Gerring, 1981). Plasma half-life is estimated to be 4-6 hours, when doses of around 4.4 mg kg<sup>-1</sup> are used. However, the plasma half-life of phenylbutazone is dose dependant at high dose rates (Piperno *et al.*, 1968; Lees *et al.*, 1985; Maitho, 1986; Lees *et al.*, 1986; Lees *et al.*, 1987). Following a single intravenous administration, the volume of distribution (V<sub>d</sub>) in the horse is low, with estimations ranging from 0.141 l kg<sup>-1</sup> (Lees *et al.*, 1987) to 0.25 l kg<sup>-1</sup> (Rose *et al.*, 1982).

Following multiple daily administrations the volume of distribution at steady state ( $V_{dss}$ ) is estimated to be  $0.152 \text{ l kg}^{-1}$  (Soma *et al.*, 1983).

Peak plasma concentrations of  $50\text{-}70 \mu\text{g ml}^{-1}$  are achieved following a single intravenous  $4.4 \text{ mg kg}^{-1}$  administration of phenylbutazone, while a similar oral dose produces peak concentrations of the order of  $8\text{-}12 \mu\text{g ml}^{-1}$  (Lees *et al.*, 1985). The time to peak plasma values ( $t_{max}$ ) following oral administration varies greatly. Concomitant access to feed generally reduces the peak plasma concentrations, and increases  $t_{max}$  (Lees *et al.*, 1983; Bogan *et al.*, 1984; Maitho *et al.*, 1986). Peak plasma concentrations are higher when larger doses are administered, and after several days of administration (Gerring *et al.*, 1981; Soma *et al.*, 1983; Crisman, 1990). Peak plasma concentrations as high as  $43.8 \mu\text{g ml}^{-1}$  have been reported following 4 days of orally administered doses of  $8.8 \text{ mg kg}^{-1}$  (Soma *et al.*, 1983). Residual plasma concentrations of  $0.2$  to  $2.0 \mu\text{g ml}^{-1}$  persist 24 hours after a single intravenous administration (about  $4.4 \text{ mg kg}^{-1}$ ), and rise to  $2\text{-}14 \mu\text{g ml}^{-1}$  after several days of the same dose (Soma *et al.*, 1985).

### Tissue fluid kinetics

The maximal phenylbutazone concentrations found in equine synovial fluid, peritoneal fluid and tissue cage fluid were always less than the maximal plasma concentrations (Lehmann *et al.*, 1981; Lees *et al.*, 1987). Concentrations one third to two thirds those of corresponding plasma concentrations reflect a relatively good penetration into tissue fluids, as well as a ready reversibility of the plasma protein binding (Lees *et al.*, 1987). However, the high degree of protein binding may explain why lower concentrations of phenylbutazone are found in synovial fluid, which has a lower protein concentration, in comparison to serum phenylbutazone concentrations (Lehmann *et al.*, 1981). In acutely inflamed joints, drug concentrations might be expected to be greater than in normal synovial fluid, due to increased blood flow, increased vascular permeability and higher extravascular protein concentrations in sites of inflammation (Higgins & Lees, 1984).

However, synovial fluid phenylbutazone concentrations in human patients treated with phenylbutazone were significantly related to plasma concentration (55-80%), but not related to plasma or synovial fluid protein concentration (Farr & Willis, 1977). Patients with more actively inflamed joints tended to have lower synovial fluid concentrations. There have been no studies reporting phenylbutazone concentrations in acutely inflamed equine joints, but concentrations of around  $4 \mu\text{g mL}^{-1}$  have been recorded from normal equine synovial fluid following single intravenously or orally administered  $4.4 \text{ mg kg}^{-1}$  doses (Lehmann *et al.*, 1981; Lees *et al.*, 1987). Due to delayed clearance, synovial fluid concentrations in an inflamed joint would not be expected to drop as quickly as plasma concentrations.

### **Inflammatory exudate kinetics**

Phenylbutazone concentrations in induced (tissue cage) acute equine inflammatory exudate approach corresponding plasma concentrations ( $12 \mu\text{g mL}^{-1}$ ) within 5 hours of intravenous administration. However, the clearance rate of phenylbutazone from acute inflammatory exudate is much slower than that from plasma, and higher concentrations of phenylbutazone could be expected to persist for longer in exudate than in plasma (Lees *et al.*, 1986). Penetration into the inflammatory exudate of more chronic lesions could be expected to be much slower and corresponding peak concentrations lower.

#### **(iii) Metabolism and elimination**

Most absorbed phenylbutazone is metabolised before being excreted, due to its lipophilic nature and the extent to which it is protein bound. Less than 4% of phenylbutazone is excreted unchanged in the urine, and the pH of equine urine is not thought to significantly affect plasma half-life (Piperno *et al.*, 1968; Smith *et al.*, 1985). Biliary excretion and possibly a degree of enterohepatic cycling are thought to occur. After intravenous and oral administration of phenylbutazone in two horses, faecal elimination accounted for 37% and 40% respectively (Smith *et al.*, 1985). Phenylbutazone is metabolised into three main products, of which only oxyphenbutazone is considered to retain significant anti-inflammatory activity. The rate of hepatic biotransformation, and

possibly biliary excretion, at any given dose, are the main factors determining plasma half-life of phenylbutazone (Tobin *et al.*, 1986).

#### (iv) Oxyphenbutazone kinetics

Oxyphenbutazone is the only metabolite of phenylbutazone that remains therapeutically active. It is less highly protein bound, and has a much higher renal clearance than phenylbutazone, but despite these factors has a similar plasma half-life (Gerken & Sams, 1988). There may be differences in the plasma protein and/or tissue binding of oxyphenbutazone at different concentrations. The  $V_d$  of oxyphenbutazone following a single intravenously administered dose of  $10 \text{ mg kg}^{-1}$  was  $0.35 \text{ l kg}^{-1}$  (Lehmann *et al.*, 1981). However, in another study, estimates of the  $V_{ds}$  for oxyphenbutazone varied from  $0.623 \text{ l kg}^{-1}$ , for a  $1.1 \text{ mg kg}^{-1}$  daily dose rate, to  $0.355 \text{ l kg}^{-1}$  for a  $4.4 \text{ mg kg}^{-1}$  daily dose rate (Gerken & Sams, 1988).

Oxyphenbutazone penetrates readily into body fluids, especially into inflammatory exudate, in which concentrations tend to be higher than plasma (Lees *et al.*, 1986). It is plausible that oxyphenbutazone concentrations may contribute to the therapeutic effect of phenylbutazone treatment (Lees *et al.*, 1987; Gerken & Sams, 1988). However, following administration of therapeutic doses of phenylbutazone, oxyphenbutazone concentrations never exceed those of phenylbutazone in any tissue fluid at any time (Lees *et al.*, 1986).

For a number of years it has been recognised that the clinical effects of phenylbutazone last longer than would be expected from its plasma half-life. The therapeutic efficacy of phenylbutazone is primarily related to the drug concentration at the site of tissue injury. Both phenylbutazone and oxyphenbutazone have a delayed clearance from inflammatory exudate. As a consequence higher concentrations of both drugs persist for longer in inflammatory exudate than in plasma. The elimination half-life of phenylbutazone from inflammatory exudate has been estimated at 24 hours, compared to a plasma elimination half-life of 4.8 hours. It has been concluded that the plasma half-lives of phenylbutazone and oxyphenbutazone can no longer be regarded as the

most important factors in determining the dose interval for phenylbutazone (Lees *et al.*, 1985).

#### 4. Clinical use

At clinically achieved concentrations, phenylbutazone is thought to selectively suppress many of the mediators of acute inflammation without adversely affecting white blood cell migration or cellular repair (Higgins *et al.*, 1984; Lees & Higgins, 1986). For these reasons it is commonly administered to control inflammation and pain associated with surgical and traumatic wounds, and is routinely used following joint surgery (McIlwraith, 1988; Vachon *et al.*, 1990; Richardson & Clark, 1990).

Large intravenous doses of phenylbutazone (10-15 mg kg<sup>-1</sup> 6 hour<sup>-1</sup>) have been used clinically to relieve the visceral pain associated with equine colic and to reduce the development of endotoxic shock. However, flunixin is much more efficacious in controlling visceral pain and attenuating the effects of endotoxic shock (Moore *et al.*, 1986; Lees *et al.*, 1987; Semrad *et al.*, 1987), and has largely replaced the use of phenylbutazone in many of these cases (Burrows, 1981; Lees *et al.*, 1985).

Phenylbutazone is still used in the management of soft tissue inflammation associated with laminitis, inflamed joints, infections, muscular damage, tendonitis, and ligamentous strains and sprains (Gabel *et al.*, 1977; Lees *et al.*, 1985; Tobin *et al.*, 1986). Soft tissue inflammation in an inflamed joint can account for more than 99% of the resistance to movement (Simon, 1981; Tobin *et al.*, 1986). Phenylbutazone results in inhibition of further swelling and facilitates less painful joint movement. Phenylbutazone is not thought to affect the progression of chronic inflammation (Higgins & Lees, 1984). The rationale for its use in chronic and degenerative conditions, such as osteoarthritis, is to reduce the clinical signs of associated concomitant episodes of acute inflammation (May *et al.*, 1987).

Regulations concerning the use of phenylbutazone have varied from total prohibition of any detectable levels to unrestricted use. Presently both the New Zealand Racing and

Harness Racing Conferences prohibit any use that results in detectable plasma concentrations at race meetings or trials, while the New Zealand Equine Federation permits plasma concentrations of phenylbutazone up to  $2 \mu\text{g ml}^{-1}$  on competition days.

## 5. Adverse effects on joints

Where regulations permit its use, phenylbutazone can be used to facilitate the training and competing of 'sore' horses (Cannon, 1973). Phenylbutazone is often used after races to prevent horses from 'cooling out sore' (Tobin, 1981; Tobin *et al.*, 1986). However, there is much debate concerning the palliative use of anti-inflammatory drugs to facilitate the continued training and racing of horses. The drug can also mask clinical signs associated with more serious injuries, which may be exacerbated by the stresses of further training or racing (Tobin *et al.*, 1986; May *et al.*, 1987; McIlwraith & Vachon, 1988). May *et al.* (1987) further states that 'use of NSAIDs in chronic lameness is likely to make prognosis worse for all other treatments except arthrodesis'.

## E. EFFECT OF PHENYLBUTAZONE ON ARTICULAR CARTILAGE

Phenylbutazone is often used for long durations to suppress inflammation and pain associated with joint damage. However, little is known on the effect of phenylbutazone on anabolic and catabolic processes occurring within normal and osteoarthritic articular cartilage.

The clinical effects of phenylbutazone have been predominantly associated with its suppression of prostanoid production (May *et al.*, 1987). In addition to the prostanoids, other chemical mediators such as leucotrienes, oxygen radicals, and a variety of cytokines such as interleukin-1 and tumour necrosis factor are involved in causing connective tissue damage associated with joint disease (Price *et al.*, 1992). Although phenylbutazone can induce a degree of local analgesia, it is unlikely that it actually prevents chronic progression of osteoarthritis in the horse (May *et al.*, 1987).

The literature is lacking in accounts of the effects of phenylbutazone on equine articular cartilage, but several studies have examined the effects of phenylbutazone on bovine and laboratory animal cartilage (Whitehouse & Haslam, 1962; Whitehouse, 1964; Bostrom *et al.*, 1964). Phenylbutazone, at a concentration of  $154 \mu\text{g mL}^{-1}$ , inhibited proteoglycan synthesis in cultured explants of bovine costal and tracheal cartilage, as measured by relative rates of incorporation of  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{14}\text{C}$ -labelled glucose, and  $^{14}\text{C}$ -labelled acetate (Whitehouse & Bostrom, 1962). Reduced proteoglycan synthesis was attributed to decreased adenosine-5- triphosphate (ATP) synthesis, due to either inhibition of cellular respiration or uncoupling of oxidative phosphorylation (Whitehouse & Haslam 1962; Bostrom *et al.*, 1964). Pulver *et al.* (1956), as reported by Domenjoz (1966), found transamination of  $\alpha$ -ketoglutaric acid with alanine to glutamic acid is competitively inhibited by phenylbutazone. Domenjoz (1966) proposed that because glutamic acid furnishes the amino groups for the biosynthesis of glucosamine and galactosamine, inhibition of its synthesis by anti-inflammatory drugs may be a limiting factor in the rate of proteoglycan synthesis.

A number of other NSAIDs can interfere with proteoglycan synthesis (Whitehouse, 1964; Bostrom *et al.*, 1964; Pالمoski & Brandt, 1979; Pالمoski *et al.*, 1980; Pالمoski & Brandt, 1983). However, the significance of these effects at clinically utilised concentrations and treatment durations remain contentious.

## F. SUMMARY

Methylprednisolone acetate and phenylbutazone are the most common steroid and non-steroidal anti-inflammatory drugs used for treatment of joint injury in equine athletes. Their soft tissue mediated clinical effects are well recognised (Higgins & Lees, 1984). Whether or not they also confer some degree of chondroprotection is actively debated (Tobin *et al.*, 1986; Burkhardt & Ghosh, 1987; McIlwraith, 1989). Furthermore, there is some evidence to suggest their use may actually potentiate the progression of joint deterioration (Whitehouse & Bostrum, 1962; Tobin *et al.*, 1986; Chunekamrai *et al.*, 1989; Trotter *et al.*, 1991; Shoemaker *et al.*, 1992). Both the types and mechanisms of their effects on articular cartilage are subjects of some conjecture and much controversy (May *et al.*, 1987; McIlwraith & Vachon, 1988; Saari *et al.*, 1992).

Relatively few controlled *in vivo* trials have sought to investigate the effects of MPA or PBZ on equine articular cartilage. Interpretation of specific drug effects from these trials has been hindered by their small sample numbers, the types of investigative procedures performed, and a range of confounding variables. The *in vitro* maintenance of tissue allows for a more controlled environment in which the study of specific interactions can be isolated from confounding variables (Tyler *et al.*, 1982). Furthermore, *in vitro* methodology facilitates the use of larger sample numbers, and more precisely controlled dose/response related investigations.

## G. OBJECTIVES

The objectives of this study were;

- (1) to set up and validate an *in vitro* tissue culture model of equine carpal articular cartilage, and
- (2) to use this model to investigate the dose related effects of phenylbutazone, and a methylprednisolone acetate formulation (Depo-Medrol®)<sup>1</sup>, on chondrocyte viability, and chondrocyte mediated degradation and synthesis of matrix proteoglycans.

## H. HYPOTHESES

The following three hypotheses were tested (with respect to chondrocyte viability, rate of proteoglycan synthesis, and proteoglycan content of the cultured explants);

- (1) the drug is capable of affecting the parameter,
- (2) the effect is apparent at clinically relevant concentrations, and
- (3) the effect is greater at higher concentrations.

## MATERIALS AND METHODS: SECTION ONE

### *DEVELOPMENT OF THE MODEL*

#### A. ANALYTICAL TECHNIQUES

##### 1. Biochemical analyses

The Gatt and Berman (1966) modification of the Elson and Morgan (1933) colorimetric method for the determination of amino sugars was to be used to estimate the glycosaminoglycan content of a large number of cartilage and media samples. It was therefore necessary to establish that (a) the assay could be performed relatively simply and without excessive variation within or between batches, and (b) glucosamine, galactosamine, chondroitin sulphate and acid digested cartilage produced similar absorption spectra.

#### Experiment 1.

##### Materials and Methods

Stock solutions of galactosamine, glucosamine and chondroitin sulphate were prepared as follows:

- (a) Deionised water was added to 0.010 g galactosamine ( $C_6O_5H_{13}N$ , 179 Da) to give 100 mL of a stock solution of  $100 \mu\text{g mL}^{-1}$  galactosamine.
- (b) Deionised water was added to 0.012 g glucosamine hydrochloride ( $C_6O_5H_{13}NHCl$ , 215.5 Da) to give 100 mL of a stock solution of  $100 \mu\text{g mL}^{-1}$  glucosamine.
- (c) Deionised water was added to 0.027 g chondroitin-6-sulphate sodium ( $C_{14}O_{14}H_{20}SNNa$ , 481 Da) to give 100 mL of a stock solution equivalent to  $100 \mu\text{g mL}^{-1}$  galactosamine.

A series of standard solutions containing 6.25, 12.5, 25, 37.5, and 50  $\mu\text{g mL}^{-1}$  of galactosamine or glucosamine were prepared by serial dilution from each stock solution. Each of these standard solutions was sealed and kept at 4°C between uses.

Three similar sized explants of equine carpal articular cartilage were placed separately into 5 mL pyrex screw-top vials containing 5 mL of 2N HCl. From each of the chondroitin sulphate standard solutions 2 mL were transferred into vials containing 1 mL of 6N HCl. Both sets of vials were sealed with teflon lined tops, placed in an oven at 100°C for 12 hours, then allowed to cool to room temperature. From each of the galactosamine and glucosamine standard solutions 2 mL were transferred into vials containing 1 mL of 6N HCl, sealed and placed in a boiling water bath for 30 minutes, then allowed to cool to room temperature.

The amino sugar content of 0.6 mL amounts from each of the vials were then assayed in triplicate according to the method of Gatt & Berman (1966), as described below.

1. Ehrlich's reagent was prepared by dissolving 1 g 4-dimethylaminobenzaldehyde in 30 mL of 1:1 ethanol:concentrated HCl (specific gravity 1.18).
2. From each of the acid digested solutions 0.6 mL amounts were transferred into 10 mL pyrex screw-top tissue culture vials, to which 0.4 mL amounts of 2M  $\text{Na}_2\text{CO}_3$  were added, followed by 0.5 mL of a 2% solution of double distilled acetylacetone in 1.5M  $\text{Na}_2\text{CO}_3$ . Reference blanks were prepared in the same way using 0.6 mL amounts of 2N HCl.
3. Each vial was gently shaken to remove excess  $\text{CO}_2$ , tightly sealed with a teflon lined screw-cap, and placed in a boiling water bath for 20 minutes.
4. The vials were allowed to cool to room temperature, and 1 mL ethanol, and 0.5 mL Ehrlich's reagent were added.
5. Each vial was shaken vigorously to expel excess  $\text{CO}_2$ , and its contents transferred to a 4.5 mL disposable polystyrene spectrophotometer cuvette<sup>1</sup>.

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<sup>1</sup> Salmond Smith Biolab N.Z. Ltd.

6. The relative absorbance of each sample was then read in a spectrophotometer<sup>2</sup>, at 524 nm, against one of the reference blanks.

The absorption spectra resulting from the three acid digested cartilage samples and the glucosamine, galactosamine and chondroitin sulphate standards were plotted for wavelengths from 440 to 600 nm. The absorbance of each of the standard solutions at 524 nm was then plotted against the amino sugar content contained in the initial 0.6 mL.

### Results and Discussion

Glucosamine and galactosamine produced different absorption spectra (Figure 2.1). The glucosamine standards consistently peaked at a slightly lower wavelength (522-524 nm as compared with 526-528 nm), and their peaks tended to be slightly higher than those of equivalent amounts of galactosamine. The intermediate wavelength of 524 nm was chosen as the wavelength which best approximated the maximal absorbances of each.

Several researchers have reported glucosamine having a greater absorbance at 530 nm than galactosamine (Balazs *et al* 1965, Gatt & Berman 1966, Blumenkrantz & Asboe-Hansen 1976). The magnitude of this difference varied between reports, and especially with slight variations of method. In addition, Gatt & Berman (1966) reported that the absorption spectrum resulting from glucosamine was of a different shape to that of galactosamine. However, none of these investigations reported that the peak absorbance of glucosamine occurs at a slightly lower wavelength than galactosamine. Furthermore, most researchers have used 530 nm as the 'peak' wavelength for the assay of either individual or combinations of amino sugars.

There was no difference in the shape of the absorption spectra between the galactosamine standards and the galactosamine released from the oven-digested chondroitin sulphate standards (Figure 2.2).

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<sup>2</sup> Pye Unicam SP8-400 UV/VIS Spectrophotometer.

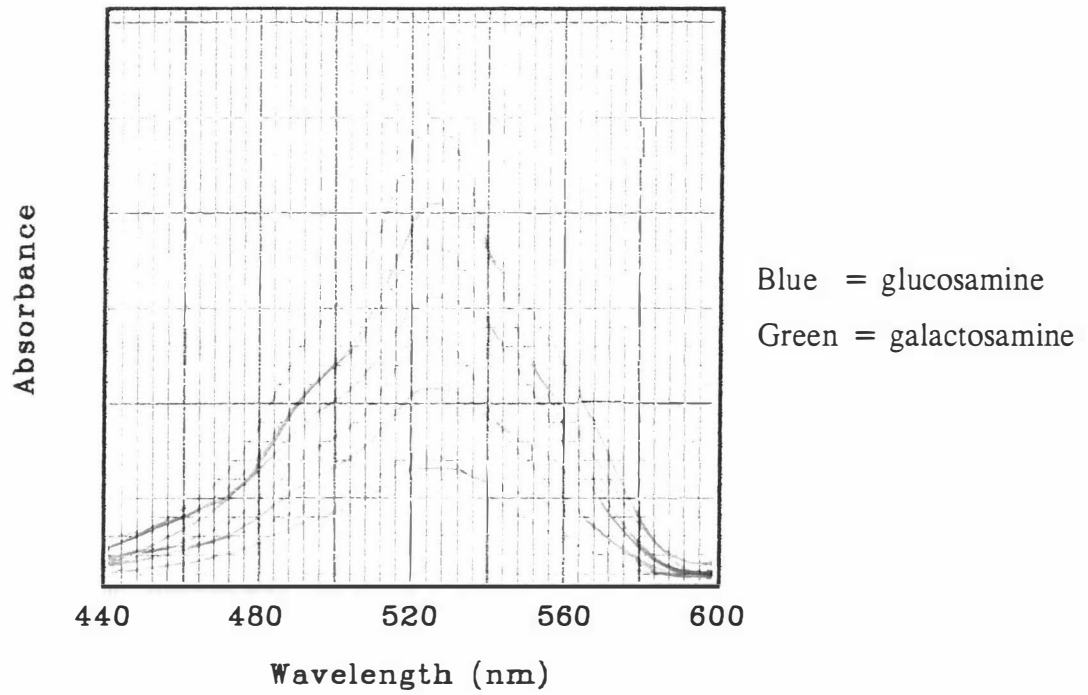


Figure 2.1 Comparison of the absorption spectra of glucosamine and galactosamine (from 5-20  $\mu\text{g}$ ).

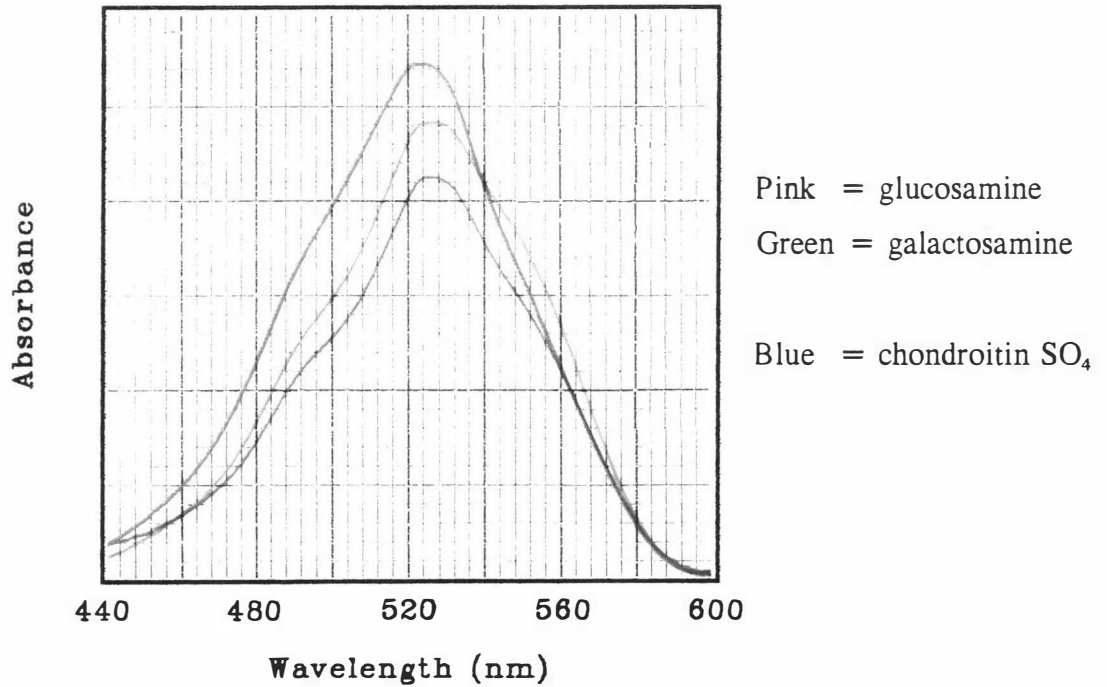


Figure 2.2 Comparison of the absorption spectra of equivalent amounts of glucosamine, galactosamine, and chondroitin sulphate.

The absorption spectra resulting from the acid digested cartilage samples (Figure 2.3) were similar in shape to those of glucosamine and galactosamine but peaked at the intermediate wavelength of 524 nm (wavelength bandwidth 2.0 Å). None of the other components of cartilage appeared to interfere with the assay.

The absorbances at 524 nm of each of the three types of standard solutions were linearly related to amino sugar amounts between 2.5-20 µg (Figure 2.4). The absorbances of the galactosamine standards were similar to those of the glucosamine standards. However, the absorbances of the chondroitin sulphate standards, which had been hydrolysed for 12 hours at 100°C, were lower than those of their theoretically equivalent galactosamine standards (Figure 2.4, Appendix Table 1).

The difference in absorbance at 524 nm produced by equal quantities of galactosamine and glucosamine was within acceptable limits for the purposes of the proposed experiments. The reduced colour yield associated with the assay of the galactosamine component of the acid digested chondroitin sulphate indicates chondroitin sulphate standards should be used in deference to galactosamine or glucosamine standards, if absolute values are to be inferred from cartilage digests. However, for comparative purposes, the use of non-digested galactosamine or glucosamine standards should be sufficient to estimate the relative amino sugar concentrations of cartilage explants.

#### **Observations made during the experiments**

- (a) The presence of any remaining effervescence greatly increased both the magnitude and variability of the absorbances recorded.
- (b) The 2% acetylacetone solution developed a faint brown discolouration, which affected the assays after several days, especially if left exposed to light.
- (c) The acetylacetone stock solution also discoloured over the course of twelve months; redistillation removed the discolouration and its associated effects.
- (d) The assay was particularly sensitive to changes in acidity resulting from any leakage during the 12 hour 100°C acid digest (Figure 2.5).

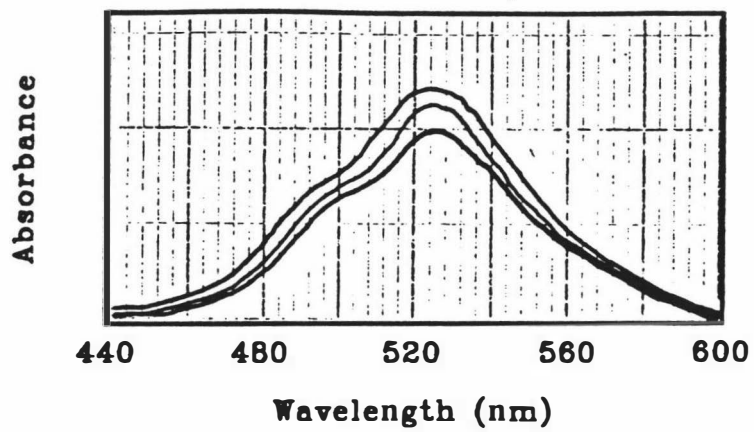
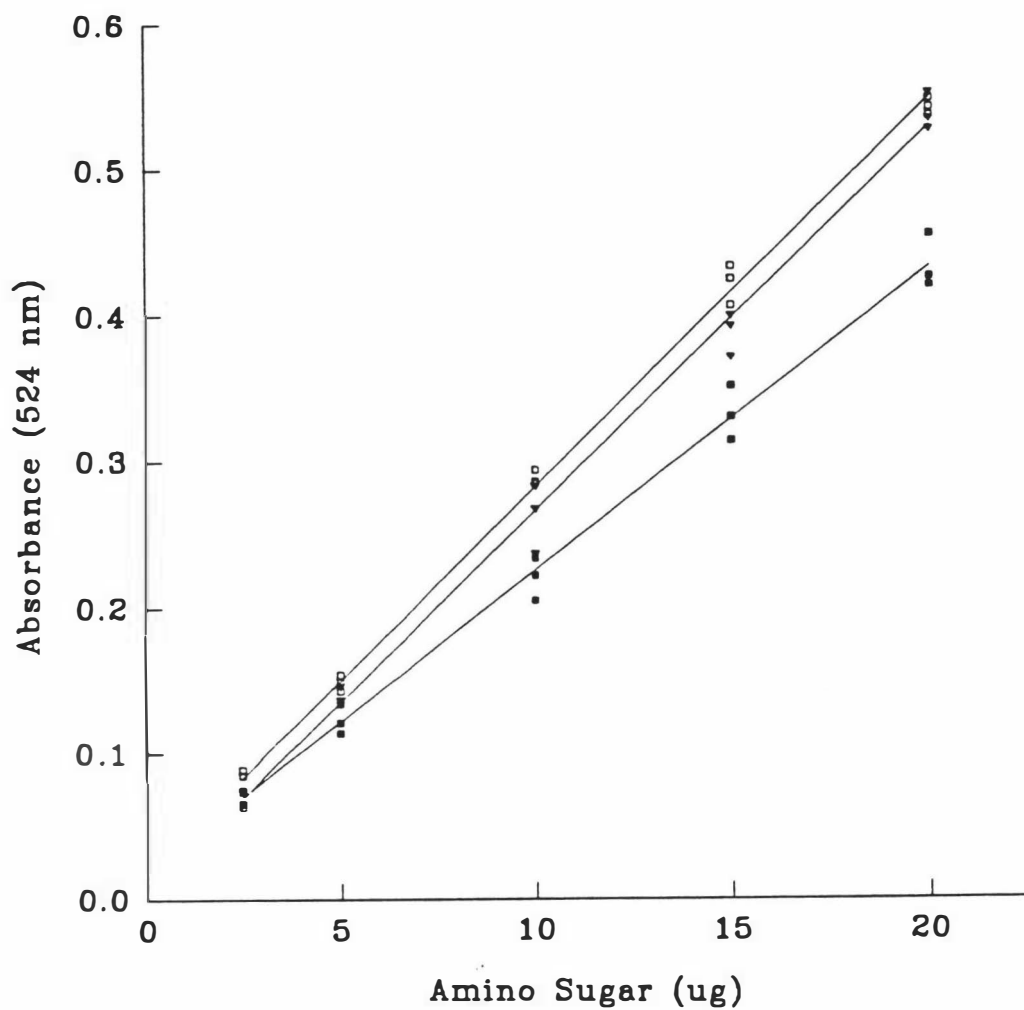
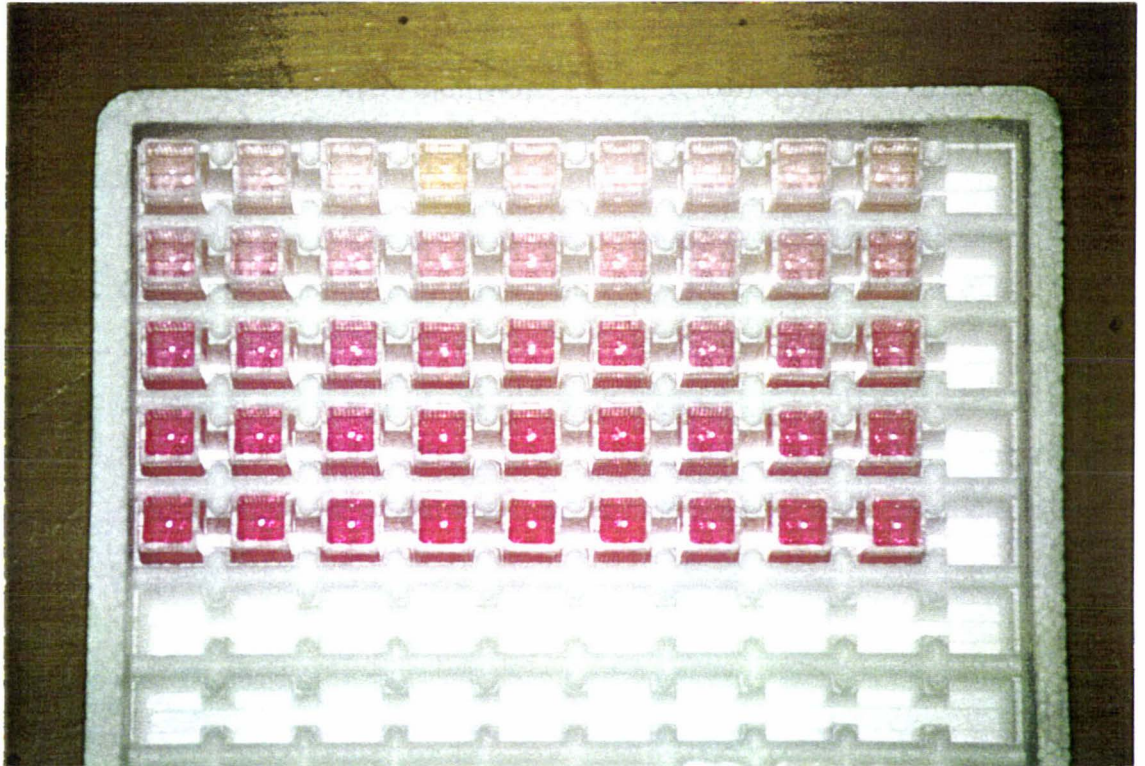


Figure 2.3 Absorption spectra of digested cartilage.



**Figure 2.4** Comparison of the standard curves of glucosamine, galactosamine, and chondroitin sulphate.

- Glucosamine (slope = 0.026, intercept = 0.004,  $r = 0.999$ )
- ▼ Galactosamine (slope = 0.026, intercept = 0.019,  $r = 0.999$ )
- Chondroitin sulphate (slope = 0.021, intercept = 0.019,  $r = 0.999$ )



**Figure 2.5** The gradation of colours produced by a range of glucosamine amounts (2.5-20  $\mu\text{g}$ ) after being assayed according to the method of Gatt & Berman. The cuvette containing the yellow/orange solution is an example of the sensitivity of the assay to any variation in acidity.

These observations were in agreement with other reports (Balazs *et al* 1965, Johnson 1971, Blumenkrantz & Asboe-Hansen 1976). Redistillation of the acetylacetone stock solution approximately every four months, and preparation of the 2% acetylacetone solution freshly on the days it is to be used, should ensure consistency of results. Vials with evidence of leakage after the oven digest should not be assayed.

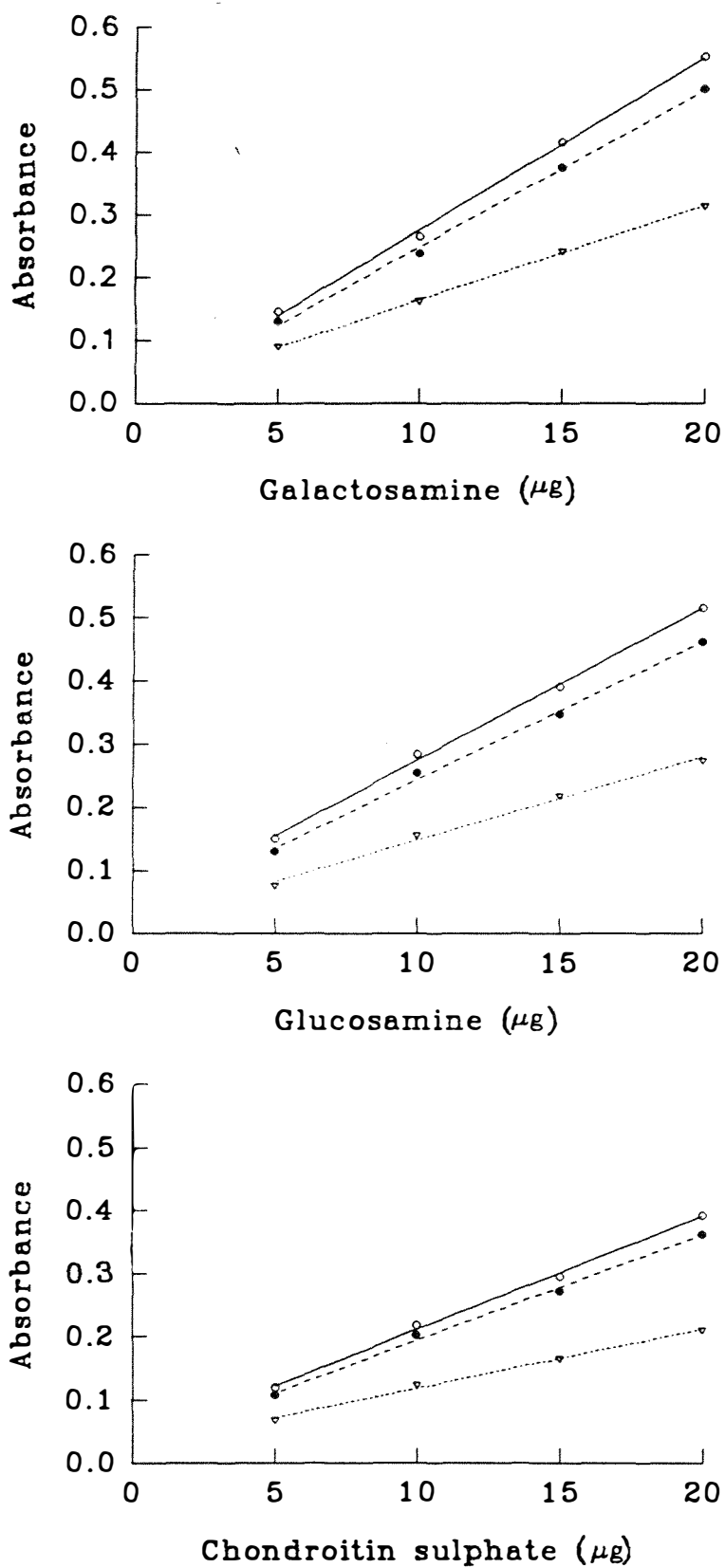
## Experiment 2.

### Materials and Methods

To determine if the stability of the assay chromophore changed with time, amounts of each of the glucosamine, galactosamine and chondroitin sulphate standard solutions were acid digested and assayed as described in Experiment 1. Absorbances at 524 nm were read within 30 minutes of the addition of Ehrlich's reagent, and then subsequently reread 2 and 23 hours later. Between readings the samples were covered and kept at room temperature.

### Results and Discussion

The results obtained from the first reading were similar to those obtained in Experiment 1 (Appendix Table 2). However, after 2 and 23 hours the absorbances had decreased to 90% and 66% respectively of the 30 minute values (Figure 2.6). The absorbance of each sample appeared to decrease in proportion to its initial value rather than by an absolute amount. These results indicate that the time between addition of the last reagent and the reading of the relative absorbance could be important, especially if groups of samples are read at different times. Intergroup variation due solely to chromophore deterioration could be minimised by processing only that number of samples which could be read in less than an hour, and by ensuring examples of each group are read at the same time.



**Figure 2.6** Variations in the amino sugar assay standard curves with time.

○ after 0.5 hour, • after 2 hours, ▽ after 23 hours

## 2. Scintillation counting

The latter stages of proteoglycan synthesis involve the addition of sulphate molecules to the glycosaminoglycan side chains. As proteoglycan synthesis is the major metabolic process using intracellular sulphate, radiolabelled sulphate incorporation has become a convenient means of studying sulphated proteoglycan synthesis. However, for the radioactivity emitted from the cartilage to be a representative measure of the rate of proteoglycan synthesis, the non-biosynthetically bound  $^{35}\text{SO}_4^{2-}$  contained within each explant must first be reduced to a minimum.

### Materials and Methods

Both carpi of an adult thoroughbred gelding, euthanased two hours previously, were skinned then carefully disarticulated at the middle carpal joint. Using a custom made cartilage chisel (Figure 2.7), full depth 4 mm wide strips of articular cartilage were harvested from the central regions of the exposed carpal bones. These were immediately placed in chilled (4 °C) Dulbecco's modified Eagle medium (DMEM)<sup>3</sup> (pH 7.4) supplemented with a cell culture antibiotic solution (PSK)<sup>4</sup>.

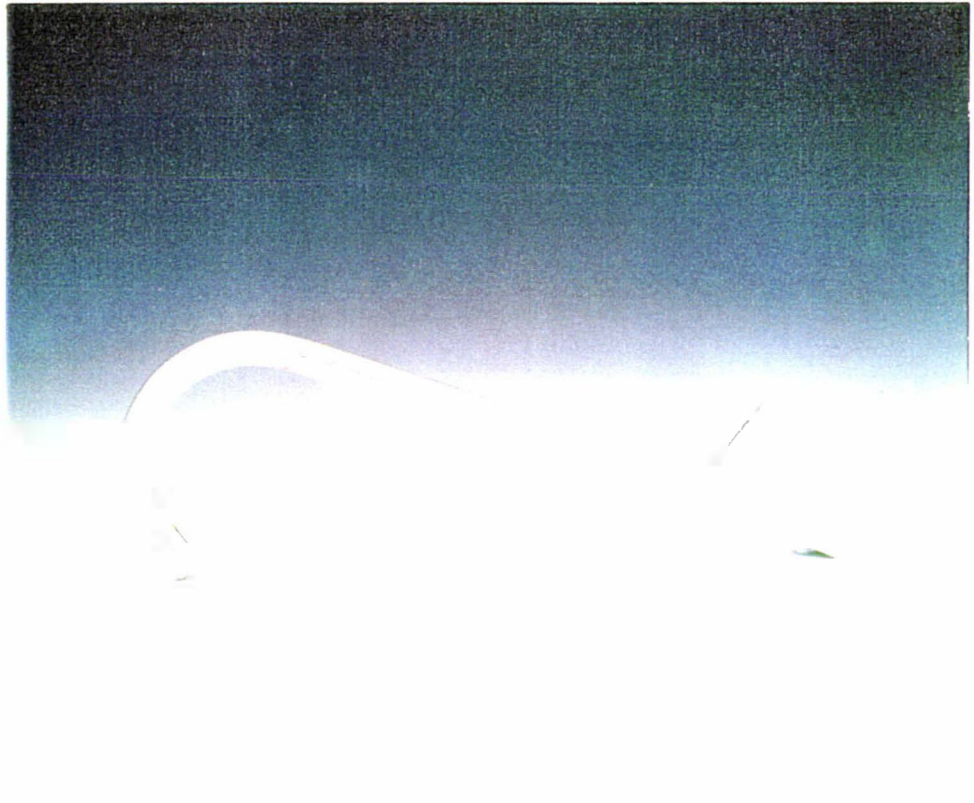
A cartilage punch with a 3 mm diameter (Figure 2.8) was used to cut 70 explants from the strips. Each explant was individually blotted dry, weighed on a Mettler balance<sup>5</sup> (reproducibility 0.05 mg), and randomly allocated into one of seven groups until each contained ten explants. The explants in group seven were frozen and stored, for later

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<sup>3</sup> Dulbecco's modified Eagle medium, high glucose (4.5 mg D-glucose per litre with glutamine but without sodium pyruvate and supplemented with 3.7 g sodium bicarbonate per litre). Gibco Laboratories, Life Technologies Inc. Grand Island, N.Y., 14072 U.S.A.

<sup>4</sup> Crystapen® injection, Glaxo N.Z. Ltd., to a final concentration of 200 IU ml<sup>-1</sup> benzylpenicillin Na. Strepomycinsulfat, Boehringer Mannheim Germany, to a final concentration of 200 µg ml<sup>-1</sup> streptomycin sulphate. Kanamycin monosulphate, Sigma Chemical Company U.S.A., to a final concentration of 150 µg ml<sup>-1</sup> kanamycin.

<sup>5</sup> Mettler AT 460 DeltaRange.



**Figure 2.7** Custom made cartilage chisel



**Figure 2.8** Cartilage punches

use as non-cultured cartilage blanks. Groups two, four and six were twice frozen (-20°C) and thawed prior to being cultured, to render the chondrocytes non-viable (Maroudas & Evans 1974).

Each explant was cultured in a tissue culture well<sup>6</sup> containing 0.5 mL of DMEM (pH 7.4), supplemented with 15% heat inactivated equine serum (ES)<sup>7</sup>, and 10  $\mu\text{Ci mL}^{-1}$   $\text{Na}_2^{35}\text{SO}_4$ <sup>8</sup>. The cultures were maintained at 37°C for 24 hours in a humidified atmosphere of 5%  $\text{CO}_2$  and air.

### Post-culture wash procedures

The explants from group one (containing live chondrocytes), group two (containing dead chondrocytes), and group seven (containing the non-cultured cartilage blanks) were each acid digested in 4 mL of 2N HCl for 12 hours at 100°C without any wash procedure.

#### (a) Two wash procedure

Each explant from group three (containing live chondrocytes) and group four (containing dead chondrocytes) was placed in 1 mL of chilled (4°C) phosphate buffered saline (pH 7.4) for one hour, then in 1 mL of chilled non-supplemented DMEM for a further hour, before being acid digested.

#### (b) Three wash procedure

Each explant from group five (containing live chondrocytes) and group six (containing dead chondrocytes) was placed in 1 mL of chilled phosphate buffered saline for 40

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<sup>6</sup> Costar 48 well cell culture cluster, Cambridge, MA 02140, U.S.A.

<sup>7</sup> Gibco's heat inactivated equine serum, Life Technologies Ltd. Penrose, Auckland New Zealand.

<sup>8</sup> Amersham Australia Pty Ltd.

minutes, then in 1 mL of chilled serum supplemented DMEM for 40 minutes, and finally in 1 mL of chilled non-supplemented DMEM for 40 minutes, before being acid digested.

### Emulsion scintillation mixture

The emulsion scintillation mixture was prepared by dissolving 4.0 g of 2,5-diphenyloxazole (PPO)<sup>9</sup>, the primary solute, and 0.1 g of 1,4di-[2,-(5-phenyloxazolyl)]benzene (POPOP)<sup>10</sup>, the secondary solute, in 667 mL of toluene<sup>11</sup> and 333 mL of triton X-100<sup>12</sup>. The mixture was then left to stand for a minimum of 24 hours to ensure dissolution of both solutes.

### Scintillation counting procedure

From each of the acid digested cartilage solutions 2 x 1 mL amounts were transferred into 20 mL glass scintillation vials containing 10 mL of the emulsion scintillation mixture. Each vial was tightly capped, thoroughly shaken until a homogenous emulsion formed, and was then left for an hour to allow suspended air bubbles to settle out.

The relative estimate of the amount of <sup>35</sup>S within each vial was made by measuring the fluorescent emissions (counts per minute) between 0 to 655 MeV in a 3 channel Beckman LS 7500 scintillation counter. Quenched standards<sup>13</sup>, and a reference background blank<sup>13</sup> were assessed at the same time.

The entire set of samples were then recounted, to confirm that the stability of the emulsions had not changed during the counting period and affected the counts per minute (cpm) recorded.

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<sup>9,10</sup>BDH laboratory reagents.

<sup>11,12</sup>Scintran<sup>®</sup>, BDH chemicals Ltd. Poole England.

<sup>13</sup> Beckman Instruments Inc. Fullerton CA 92634 U.S.A.

### Conversion of cpm to degenerations per minute (dpm)

Counter efficiency (CE) values for the quenched standards were calculated by dividing the cpm measured by the theoretically occurring dpm.

$$CE_{\text{standards}} = \frac{\text{cpm}}{\text{dpm}}$$

These counter efficiency values were plotted against the H numbers of the quenched standards (Figure 2.9 and Appendix Table 3), and the resulting 'quench curve' was then used to estimate the CE value for each sample from its own H number. The dpm of the non-cultured cartilage blanks ( $\text{dpm}_{\text{blank}}$ ) were calculated by dividing each cpm value by its estimated CE value.

$$\text{dpm}_{\text{blank}} = \frac{\text{cpm}_{\text{blank}}}{CE_{\text{blank}}}$$

The dpm values of the blanks were then averaged to provide an estimate of the background radiation ( $\text{dpm}_{\text{background}}$ ). The cpm from each vial were converted into dpm by dividing them by their calculated counter efficiency values and subtracting the estimate of the background radiation from this total.

$$\text{dpm} = \frac{\text{cpm}}{CE} - \text{dpm}_{\text{background}}$$

The two dpm ( $\text{dpm}_{\text{rep1}}$  and  $\text{dpm}_{\text{rep2}}$ ) values for each sample were then averaged, divided by the weight of the explant (wgt), and multiplied by four (the dilution factor) to give the  $\text{dpm mg}^{-1}$  of articular cartilage.

$$\text{dpm mg}^{-1} = \frac{4(\text{dpm}_{\text{rep1}} + \text{dpm}_{\text{rep2}})}{2\text{wgt}}$$

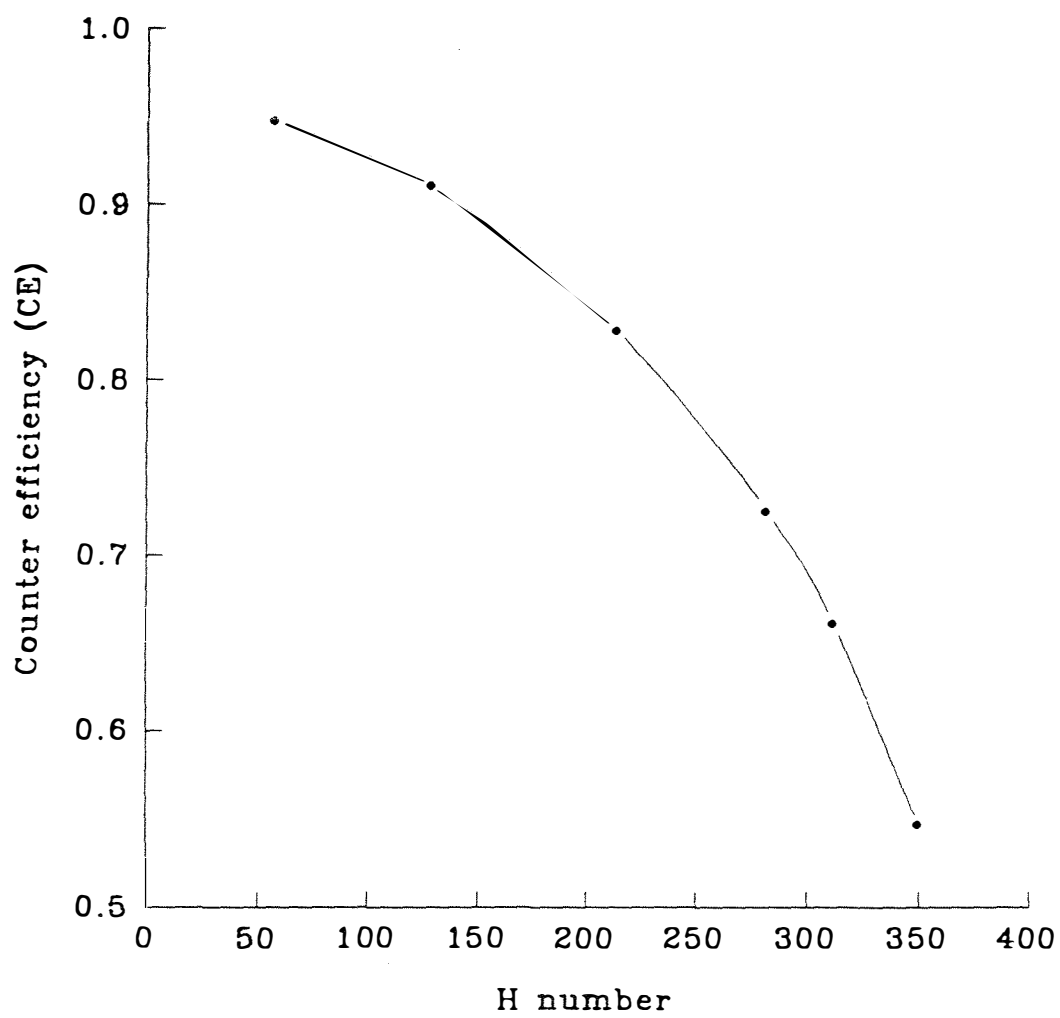


Figure 2.9 Quench curve generated by  $^{14}\text{C}$  quenched standards.

The percentage of the radioactivity in unwashed explants not due to biosynthetically bound  $^{35}\text{S}$  was estimated by;

$$1. \quad \frac{\text{average dpm mg}^{-1} \text{ for group two (dead chondrocytes)}}{\text{average dpm mg}^{-1} \text{ for group one (live chondrocytes)}} \times \frac{1}{100}$$

The percentage of the radiolabel not biosynthetically bound and still remaining after two washes was estimated by;

$$2. \quad \frac{\text{average dpm mg}^{-1} \text{ for group four (dead chondrocytes)}}{\text{average dpm mg}^{-1} \text{ for group three (live chondrocytes)}} \times \frac{1}{100}$$

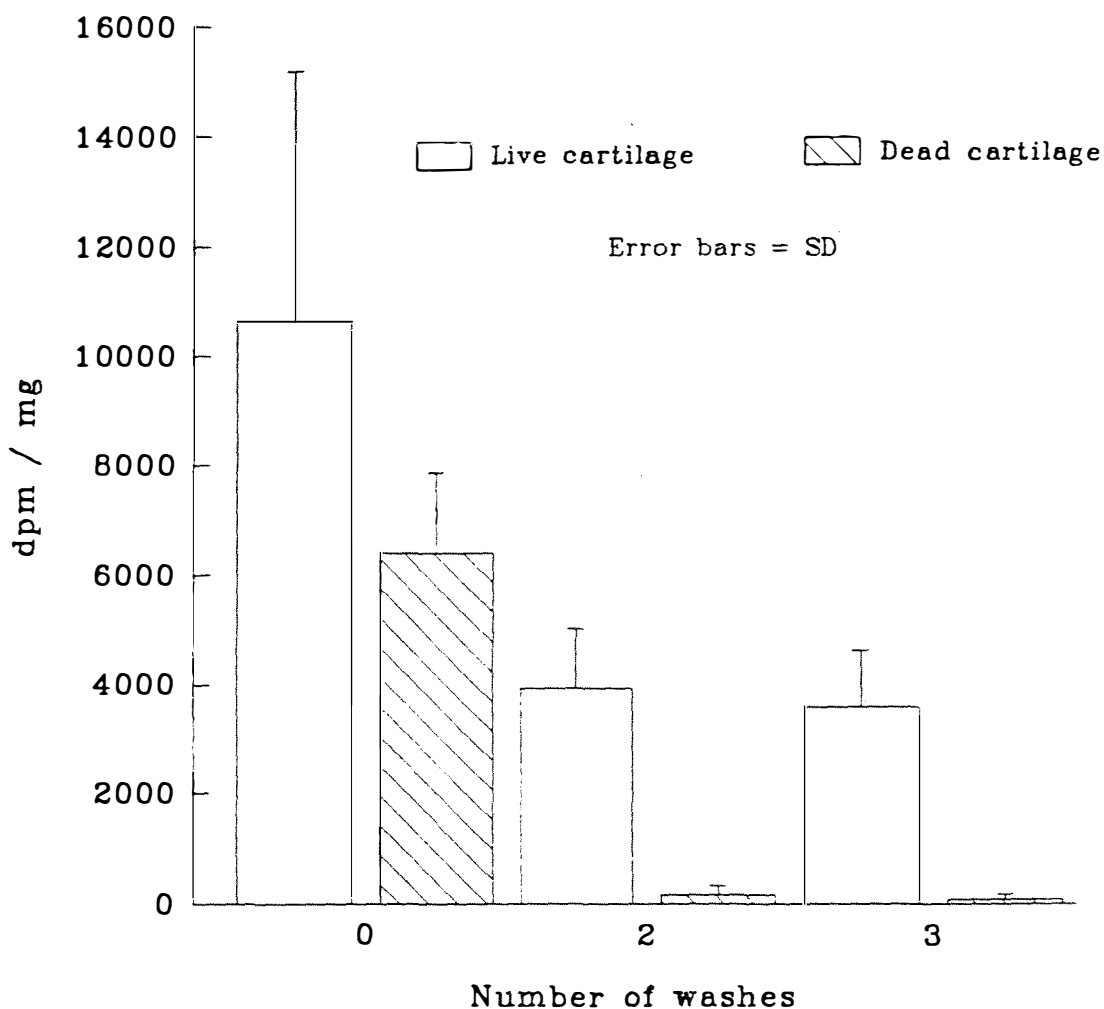
The percentage of radiolabel not biosynthetically bound and still remaining after three washes was estimated by;

$$3. \quad \frac{\text{average dpm mg}^{-1} \text{ for group six (dead chondrocytes)}}{\text{average dpm mg}^{-1} \text{ for group five (live chondrocytes)}} \times \frac{1}{100}$$

## Results and Discussion

Both the two wash and the three wash technique were effective in reducing the non-biosynthetically incorporated radiolabel to less than 5% of the incorporated level (Figure 2.10 and Appendix Table 4). The three wash technique appeared slightly more effective, but the difference was not statistically significant ( $\alpha=0.05$ ).

Theoretically, each wash potentially represented a 1:200 dilution of the radiolabel. However, radiolabel concurrently transferred on the surfaces of the explant and forceps may have reduced this dilution factor. The permeability coefficients of sulphate ion in post-mortem human articular cartilage have been found to be the same whether cells are alive or dead; thus the diffusion of sulphate is not via active transport (Maroudas &



**Figure 2.10** Comparison of wash procedures on biologically incorporated versus non-bound  $^{35}\text{S}$  content of explants of equine articular cartilage.

Evans 1974). The time taken for radiolabelled sulphate to equilibrate with the pools of non-labelled sulphate ions is thought to primarily depend on its rate of diffusion through the cartilage matrix.  $^{35}\text{SO}_4^{2-}$  has been calculated to achieve 90% of its final equilibrium concentration across a 1 mm plug of articular cartilage within 6 minutes (Maroudas & Evans 1974). Thus, neither the volume of the wash solutions, nor the time each explant was exposed to them should have been a limiting factor in this experiment. The relatively large counts from some of the washed 'non-viable explants' may have been the result of some cells still remaining viable despite being frozen and thawed twice.

The use of a suction pipette to remove the radiolabelled media and wash solutions prior to removal of each explant should minimise concurrent transfer of excess radiolabel on the surfaces of the explants or forceps. The three wash technique, which includes a wash in serum supplemented media, should also further help to reduce the concentration of protein bound drugs in the explants.

### 3. DNA Assay

Inter-sample variation was to be reduced by expressing the  $^{35}\text{S}$  incorporation (dpm) and amino sugar content of each explant as a function of both its wet weight and DNA content. It was therefore necessary to establish that the DNA assay could be performed relatively simply and without excessive variation within or between batches.

#### Materials and methods

Pyrex vials (5 mL) were prewashed in nitric acid and rinsed in distilled deionised water to exclude extraneous sources of DNA. Explants of equine articular cartilage were placed into vials containing 0.05 mL papain<sup>14</sup> and 0.95 mL phosphate buffered saline (PBS). Each vial was sealed and incubated at 60°C for 12 hours, then allowed to cool to room temperature.

Standard solutions containing between 2-10  $\mu\text{g mL}^{-1}$  DNA were prepared by serially diluting a liquid preparation of calf DNA<sup>15</sup> with sterile PBS (0.05 M  $\text{NaPO}_4$ , 2 M NaCl, pH 7.4). The DNA content of the digests and standards were assayed according to the procedure of Labarca & Paigen (1980).

1. The H33258 reagent solution was made by adding Hoechst 33258<sup>16</sup> to PBS to a final concentration of 1  $\mu\text{g mL}^{-1}$ .
2. Each cartilage digest and each standard solution was briefly sonicated immediately prior to being assayed.
3. Using a small bore gel loading pipette tip, 0.05 mL from each of the cartilage digests and standard solutions was removed and added to acrylic fluorometric cuvettes containing 2 mL of the H33258 reagent solution (in triplicate). These

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<sup>14</sup> Stabilised liquid papain, Salmond Smith Biolab Ltd.

<sup>15</sup> Calf DNA, Boehringer Mannheim N.Z. Ltd.

<sup>16</sup> Hoechst 33258, (2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol.3HCl, BrdU, bromodeoxyuridine.

were capped and inverted several times, to ensure even mixing, and then allowed to settle for 30 minutes.

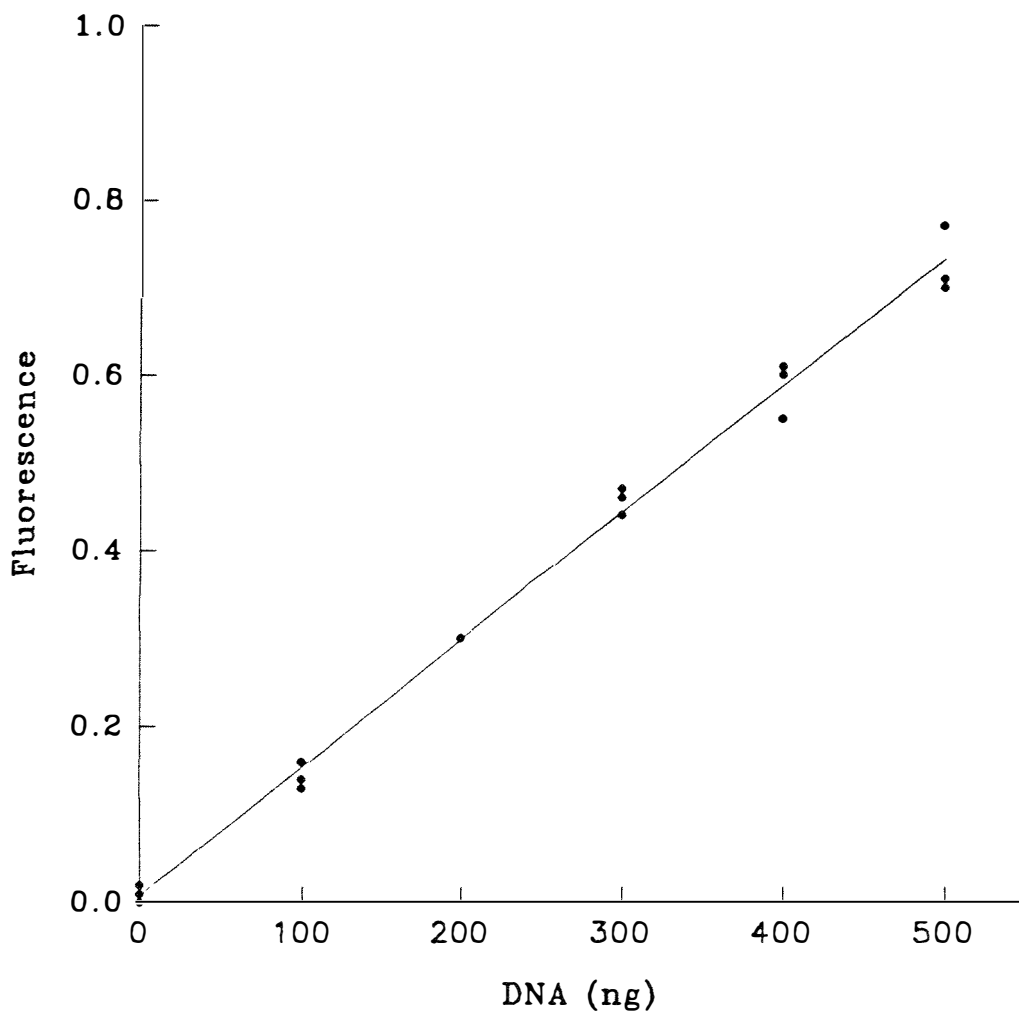
4. Fluorescence was measured in a fluorometer with the excitation wavelength set at 356 nm and the emission wavelength set at 458 nm.

### Results and Discussion

The 0.05 mL amounts of the DNA standard solutions contained 100, 200, 300, 400, and 500 ng of DNA. Fluorescence was linearly related to the amount of DNA with little variation between replicates (Figure 2.11 & Appendix Table 5). The DNA content of the digested cartilage aliquots ranged from 64.1-166.7 ng (mean = 120.9, SD = 23.8 ng). However, there was substantial variation between the replicates of the digested cartilage samples Table 2.1. This variation could have been caused by either a lack of homogeneity in the digested cartilage solutions or problems with assay technique and or equipment. Either way the results indicated further assessment of this assay would be necessary before it could be included in the final experimental protocol.

Sample	Rep 1	Rep 2	Rep 3	Mean	DNA
	(Relative fluorescence)				(ng)
<b>1</b>	0.10	0.19	0.16	<b>0.15</b>	<b>98.3</b>
<b>2</b>	0.24	0.17	0.25	<b>0.22</b>	<b>146.2</b>
<b>3</b>	0.20	0.21	0.14	<b>0.18</b>	<b>118.8</b>
<b>4</b>	0.13	0.18	0.18	<b>0.16</b>	<b>105.2</b>
<b>5</b>	0.20	0.13	0.16	<b>0.16</b>	<b>105.2</b>
<b>6</b>	0.17	0.11	0.19	<b>0.16</b>	<b>105.2</b>
<b>7</b>	0.20	0.15	0.19	<b>0.18</b>	<b>118.8</b>
<b>8</b>	0.18	0.12	0.18	<b>0.16</b>	<b>105.2</b>
<b>9</b>	0.15	0.18	0.18	<b>0.17</b>	<b>112.0</b>
<b>10</b>	0.19	0.20	0.18	<b>0.19</b>	<b>125.0</b>

**Table 2.1 DNA assay of digested cartilage samples**



**Figure 2.11** Standard curve for calf thymus DNA assayed according to the method of Labarca and Paigen (1980).

## **B. MEASUREMENT OF PROTEOGLYCANS IN THE CULTURE MEDIA**

### **1. Factors affecting the amino sugar assay**

It was intended to measure the amount of proteoglycan released from each explant into the medium during culture. It was therefore necessary to establish whether the culture medium, drugs being tested, or serum interfered with the amino sugar assay.

#### **Experiment 1.**

##### **Materials and Methods**

1. DMEM was added to 0.0120 g glucosamine hydrochloride to a final volume of 100 mL to give a stock solution of  $100 \mu\text{g mL}^{-1}$  glucosamine in DMEM. Standard solutions containing 0, 12.5, 25, 37.5, and  $50 \mu\text{g mL}^{-1}$  glucosamine were prepared by serially diluting this stock solution with DMEM.
2. From each of these standard solutions 0.4 mL amounts were mixed with 0.2 mL 6N HCl, and then assayed in triplicate for amino sugar content according to the method of Gatt & Berman (1966) as described earlier.
3. In addition, 1.5 mL of each standard solution was added to a 5 mL pyrex vial containing 0.5 mL 6N HCl. These were sealed, placed in a  $100^\circ\text{C}$  oven for 12 hours, then 0.6 mL amounts were assayed in triplicate for amino sugar content.

##### **Results and Discussion**

The absorption spectra from the glucosamine standards dissolved in DMEM (Figure 2.13 and Appendix Table 7) shared some similarity to those obtained from glucosamine standards dissolved in water (Figure 2.12 and Appendix Table 6), but were more variable and contained an extra peak at approximately 556 nm. In contrast, the absorption spectra from the oven-digested glucosamine standards (Figure 2.14) had a different shape, were much more variable, and were less representative of their glucosamine content.

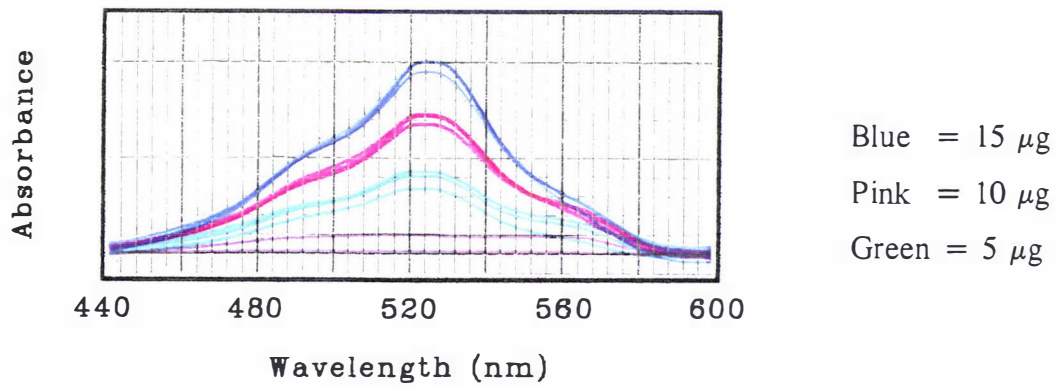


Figure 2.12 Absorption spectra of glucosamine standards dissolved in water.

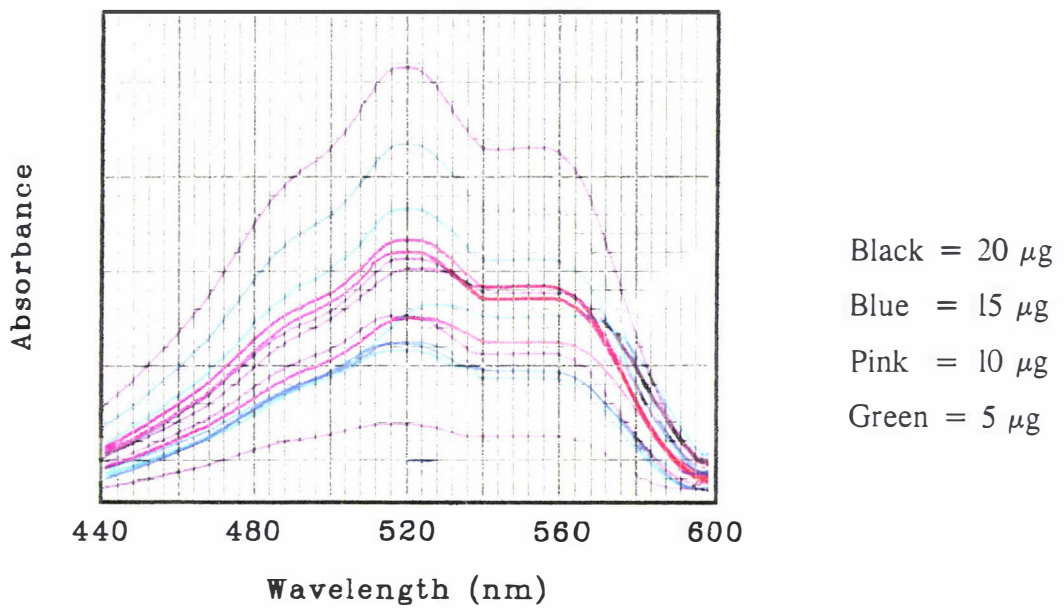


Figure 2.13 Absorption spectra of glucosamine standards dissolved in DMEM.

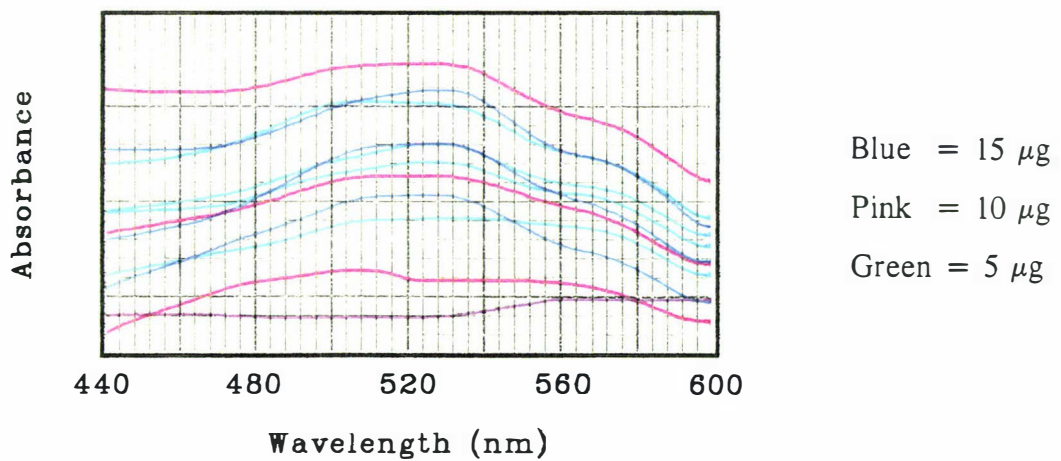


Figure 2.14 Absorption spectra of glucosamine standards dissolved in DMEM and assayed after oven digestion in 2N HCl.

The absorption spectra from the non-oven-digested standards indicated that at least one of the components of DMEM resulted in a chromophore which directly interfered with the assay of glucosamine. In comparison, the absorption spectra from the oven-digested standards suggested acid digestion of the medium resulted in a product or products which interfered with the assay of glucosamine. The results were not unexpected considering the experiment attempted to assay microgram amounts of amino sugar from a culture medium containing milligram amounts of a variety of sugars and amino acids (Table 2.2).

## Experiment 2.

Culture media used for explant culture are usually supplemented with serum. The aim of this study was to ascertain if equine serum (ES) interfered with the assay of standard amounts of glucosamine.

### Materials and Methods

1. The stock solution containing glucosamine dissolved in DMEM ( $100 \mu\text{g mL}^{-1}$ ) was further diluted with DMEM and ES to make standard solutions of 0, 12.5, 25, 37.5, and  $50 \mu\text{g mL}^{-1}$  glucosamine in DMEM+ES.
2. From each of the standard solutions 1.5 mL were added to 5 mL pyrex vials containing 0.5 mL 6N HCl. These were sealed and kept at  $100^\circ\text{C}$  for 12 hours, before 0.6 mL amounts were assayed in triplicate for amino sugar content.

### Results and Discussion

The formation of black suspended particles during the oven digestion precluded assay of the serum supplemented glucosamine standards. These suspended particles, thought to be partially oxidised serum protein, caused scatter and prevented the reading of a meaningful absorbance.

<b>Components</b>	<b>Concentrations (mg l<sup>-1</sup>)</b>
Glucose	4500
Phenol red	15
L-Arginine.HCl	84
L-Cystine.HCl	63
L-Glutamine	584
Glycine	30
L-Histidine.HCl.H <sub>2</sub> O	42
L-Isoleucine	105
L-Leucine	105
L-Lysine.HCl	146
L-Methionine	30
L-Phenylalanine	66
L-Serine	42
L-Threonine	95
L-Tryptophan	16
L-Tyrosine 2Na.2H <sub>2</sub> O	104
L-Valine	94
D-Ca Pantothenate	4
Choline chloride	4
Folic acid	4
i-Inositol	7.2
Niacinamide	4
Pyridoxal.HCl	4
Riboflavin	0.4
Thiamine.HCl	4
CaCl <sub>2</sub> (anhydrous)	200
Fe(NO <sub>3</sub> ).9H <sub>2</sub> O	0.1
KCl	400
MgSO <sub>4</sub> (anhydrous)	98
NaCl	6400
NaHCO <sub>3</sub>	3700
NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	125

Bovine albumin (500  $\mu\text{g}$ ) had previously been reported not to interfere with the detection of galactosamine from chondroitin sulphate (20  $\mu\text{g}$ ) in samples acid digested for 30 minutes at 120°C (Blumenkrantz & Asboe-Hansen 1976). In contrast to the experiments performed by Blumenkrantz & Asboe-Hansen (1976), each sample assayed in this experiment contained approximately 3600  $\mu\text{g}$  of equine serum albumin, plus the other components of serum, and was digested for 12 hours at 100°C.

### Experiment 3.

It was also possible that either the actual drugs to be tested, or a component of their formulations, could interfere with the assay. The aim of this experiment was to determine whether common formulations of the drugs likely to be used on the explant model directly affected the assay.

#### Materials and methods

1. Aqueous solutions were prepared which contained (a) 100  $\mu\text{g mL}^{-1}$  phenylbutazone sodium<sup>17</sup>, (b) 1000  $\mu\text{g mL}^{-1}$  betamethasone sodium phosphate<sup>18</sup>, (c) 1000  $\mu\text{g mL}^{-1}$  gentamicin<sup>19</sup> and (d) 50  $\mu\text{g mL}^{-1}$  flunixin meglumine<sup>20</sup>.
2. From each of these solutions 1.5 mL were added to 5 mL pyrex vials containing 0.5 mL 6N HCl, which were sealed and kept at 100°C for 12 hours, then 0.6 mL amounts were assayed for amino sugar content, and the resulting absorption spectra plotted for wavelengths from 440-600 nm.

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<sup>17</sup> Butazone, Phenylbutazone sodium, C-Vet Ltd.

<sup>18</sup> Betsolan Soluble, Betamethasone sodium diphosphate, Pitman-Moore.

<sup>19</sup> Gentamicin 50, Gentamicin sulphate, R.W.R Veterinary Products Ltd.

<sup>20</sup> Finadyne, Flunixin Meglumine, Schering Corporation.

## Results and Discussion

The peak absorbance (524 nm) of the gentamicin solution was many times greater than the peak absorbances of the glucosamine standards previously assayed. The result was not unexpected considering gentamicin contains amino sugars as part of its molecular structure. The phenylbutazone formulation resulted in the formation of an orange precipitate during the assay procedure, precluding the spectrophotometric reading of a meaningful absorbance. However, the betamethasone and flunixin meglumine solutions had comparatively small absorbances at 524 nm, and the shape of the absorption spectrum of each was different to that of glucosamine.

### 2. Methods to reduce media and drug interference

The results from the previous three experiments indicated that further processing was needed to remove or reduce media, serum and drug concentrations before the amino sugar content of proteoglycans released during culture could be assayed.

#### Experiment 1.

A number of the components of DMEM may have contributed to its interference of the assay of amino sugar standards. Selective omission of certain media components and aqueous dilution were thus investigated as means of reducing this interference.

#### Materials and Methods

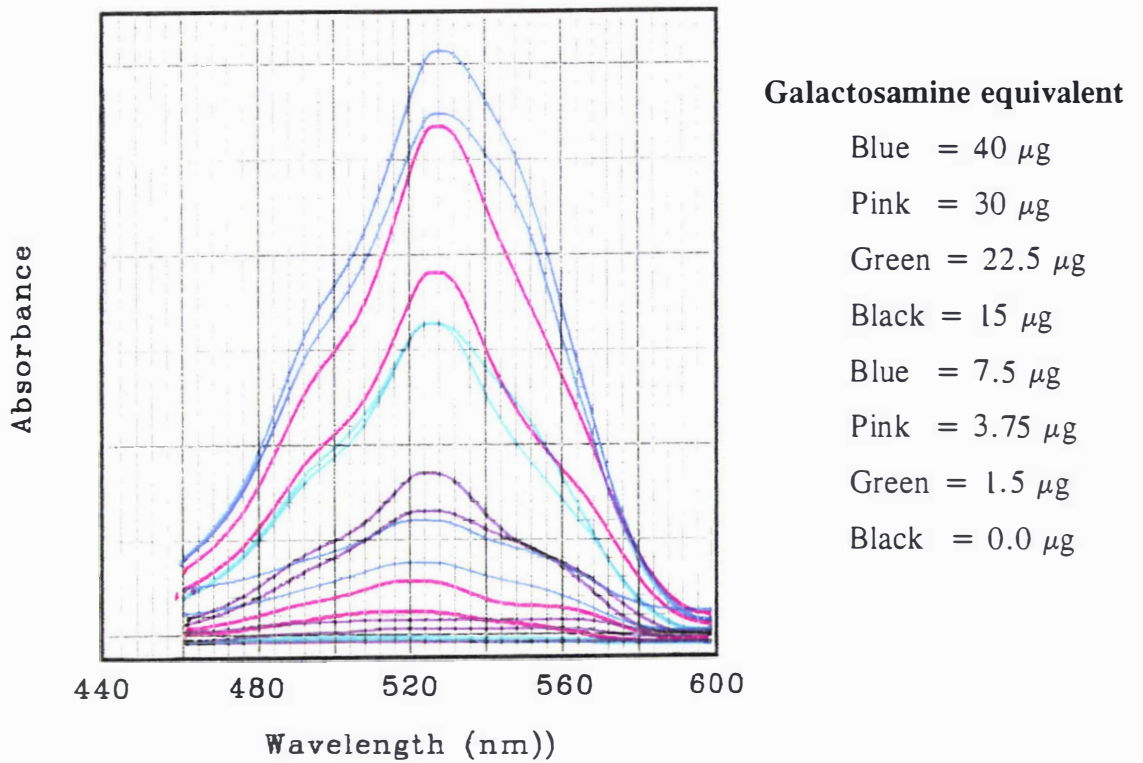
1. A stock solution of chondroitin-6-sulphate in DMEM, equivalent to  $500 \mu\text{g mL}^{-1}$  galactosamine, was prepared by adding DMEM to 0.135 g chondroitin-6-sulphate sodium ( $\text{C}_{14}\text{O}_{14}\text{H}_{20}\text{SNNa}$ , 481 Da) to give a final volume of 100 mL.
2. The chondroitin sulphate stock solution was serially diluted with volumes of DMEM to produce a series of solutions containing the equivalent of 225, 375 and  $500 \mu\text{g mL}^{-1}$  galactosamine in DMEM. Each solution was then diluted 1:9 with water to give chondroitin-6-sulphate standards, dissolved in 10% DMEM,

which theoretically contained the equivalent of 18.75, 37.5, and 50  $\mu\text{g mL}^{-1}$  galactosamine.

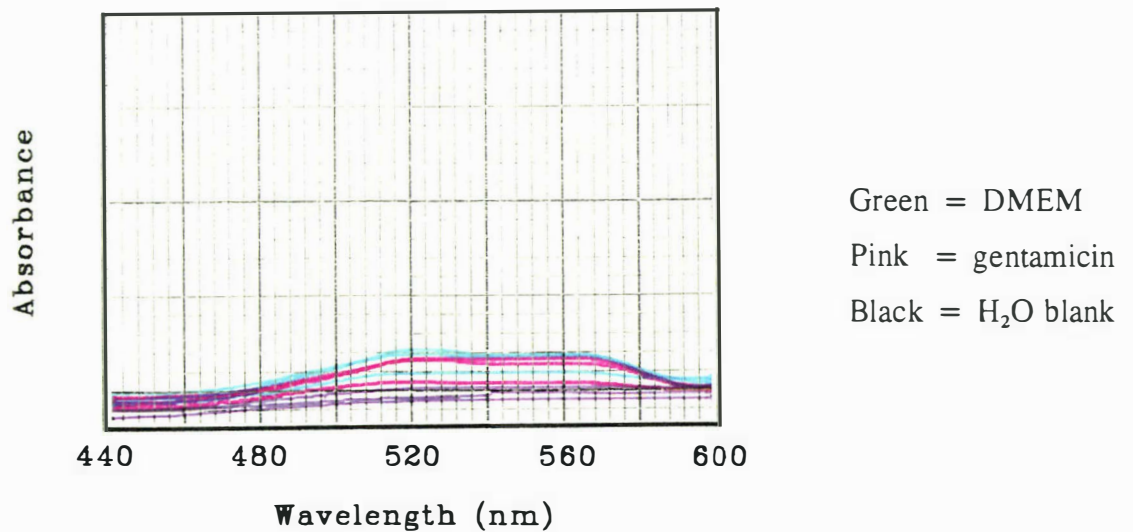
3. Phenol red was added to 100 mL of deionised water (pH 7.4) until the colour of the resulting solution approximated that of DMEM (pH 7.4).
4. Two further solutions of DMEM were prepared; (a) DMEM supplemented with L-glutamine (2000  $\mu\text{g mL}^{-1}$ ), and (b) DMEM diluted 1:4 with water.
5. From each of the chondroitin-6-sulphate standard solutions 1.5 mL were added to 5 mL pyrex vials containing 0.5 mL 6N HCl, which were sealed and kept at 100°C for 12 hours, then 0.6 mL amounts of each were assayed for their amino sugar content.
6. Amounts (1.5 mL) of the (a) aqueous phenol red solution, (b) DMEM, (c) DMEM supplemented with L-glutamine, and (d) 1:4 aqueous dilution of DMEM were added to 5 mL pyrex vials containing 0.5 mL of 6N HCl. These vials were sealed and placed in a boiling water bath for 30 minutes, then 0.6 mL amounts were assayed for amino sugar content according to the method of Gatt & Berman (1966), with the exception that their absorption spectra were plotted relative to a non-assayed distilled water blank.

## Results and Discussion

The use of water blanks instead of assayed blanks resulted in larger more readily comparable absorbances at 524 nm. DMEM had a similar absorption spectrum to glucosamine and galactosamine, but had an additional peak at around 564 nm (Figure 2.15). There was no difference between the absorption spectra of DMEM and DMEM supplemented with L-glutamine. The aqueous phenol red solution had a single small absorption peak at approximately 508 nm, in comparison to the dual peaks at approximately 524 and 564 nm produced by DMEM (Figure 2.15), indicating phenol red was not the most important component of DMEM interfering with the assay of amino sugars. The absorption spectra of the oven-digested chondroitin-6-sulphate standards dissolved in a 10% aqueous solution of DMEM were similar to chondroitin-6-sulphate standards dissolved in water.



**Figure 2.15** Absorption spectra of chondroitin sulphate standards dissolved in DMEM following dialysis and oven digestion in 2N HCl (2.0 A).



**Figure 2.16** Absorption spectra of DMEM and gentamicin following dialysis and oven digestion in 2N HCl (relative to a water blank, 2.0 A).

The results indicate little advantage would be gained by excluding either L-glutamine or phenol red from the DMEM formulation. However, the use of processing techniques which selectively dilute the culture medium with respect to the proteoglycans could greatly reduce the interference the culture medium had on this assay.

## Experiment 2.

Dialysis was investigated as a means of reducing the interference caused by either culture media components or supplemented drugs, in the assay of the amino sugar component of chondroitin sulphate standards .

### Materials and methods

1. Dialysis tubing<sup>21</sup> was prepared by boiling it in 0.05 mol l<sup>-1</sup> Na<sub>2</sub>CO<sub>3</sub> and 0.01 mol l<sup>-1</sup> sodium ethylenediaminetetra-acetic acid (EDTA) for 10 minutes, followed by 10 minutes in boiling deionised water, with a final rinse in cold deionised water. The tubing was then cut into 5 cm lengths, and one end was folded over and clamped to form open ended sacks.
2. Portions of the chondroitin sulphate in DMEM stock solution (500 µg mL<sup>-1</sup> galactosamine in DMEM) were diluted with DMEM to produce a series of standard solutions containing the equivalent of 0-100 µg mL<sup>-1</sup> galactosamine in DMEM.
3. Portions of the chondroitin sulphate in water stock solution (100 µg mL<sup>-1</sup> galactosamine) were diluted with water to produce aqueous standard solutions containing the equivalent of 0-100 µg mL<sup>-1</sup> galactosamine.
4. From each of the two sets of standard solutions 1 mL volumes were placed in dialysis sacks which were sealed and suspended for 24 hours in three changes of deionised water at 4 °C, each equivalent to 100 times the combined volume of the samples.

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<sup>21</sup> Union Carbide 10 mm cellulose dialysis membrane, molecular weight cut off approximately 12-20 kDa.

5. Volumes (1 mL) of (a) DMEM, (b) water, and (c) a  $1000 \mu\text{g mL}^{-1}$  aqueous solution of gentamicin were transferred into dialysis sacks in triplicate and individually dialysed for 24 hours against two changes of deionised water ( $4^\circ\text{C}$ ).
6. The contents of the dialysed sacks were emptied into graduated vials, standardised to 1.2 mL, and 1 mL transferred to 5 mL pyrex vials containing 0.5 mL 6N HCl. These vials were sealed and placed in an oven kept at  $100^\circ\text{C}$  for 12 hours, then 0.6 mL amounts were assayed in duplicate for amino sugar content, and the absorption spectra plotted for wavelengths from 440-600 nm.

### Results and Discussion

Chondroitin sulphate was used instead of glucosamine as its molecules were more likely to be retained within the dialysis sacks because of their larger size. The molecular weight of the chondroitin sulphate used was not known, but the average molecular weight of chondroitin sulphate molecules extracted from articular cartilage has been estimated to be around 20 kDa (Stockwell, 1979; Carney & Osborne, 1991). The molecular weight cut-off range for the dialysis tubing used was 12-20 kDa. Pore sizes of dialysis membranes are not uniform, but can be expected to have a normal distribution around the mean pore size. Boiling of the dialysis tubing prior to its use reportedly ensures a more uniform pore size.

Dialysis removed much of the interference caused by DMEM in the assay of the galactosamine component of the chondroitin sulphate standards (Figures 2.16 & 2.18). The use of dialysis resulted in only small losses of chondroitin sulphate but a dramatic reduction in the concentration of both DMEM components and gentamicin left within each sack (Figure 2.17). However, the need to restandardise the volume of each dialysed sample coupled with the extra number of sample manipulations associated with the use of dialysis could be expected to increase the overall experimental error, and produced more variable results.

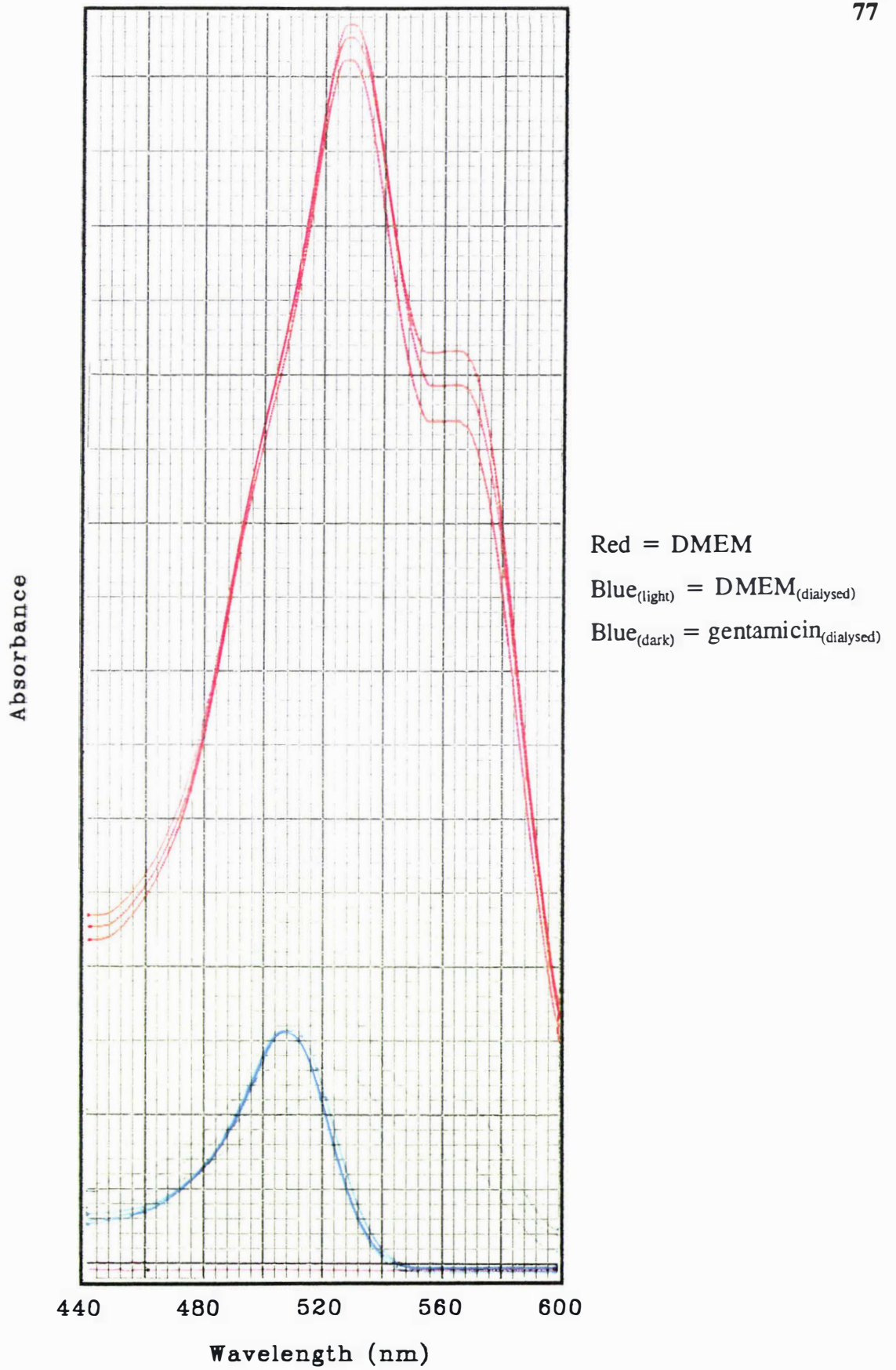
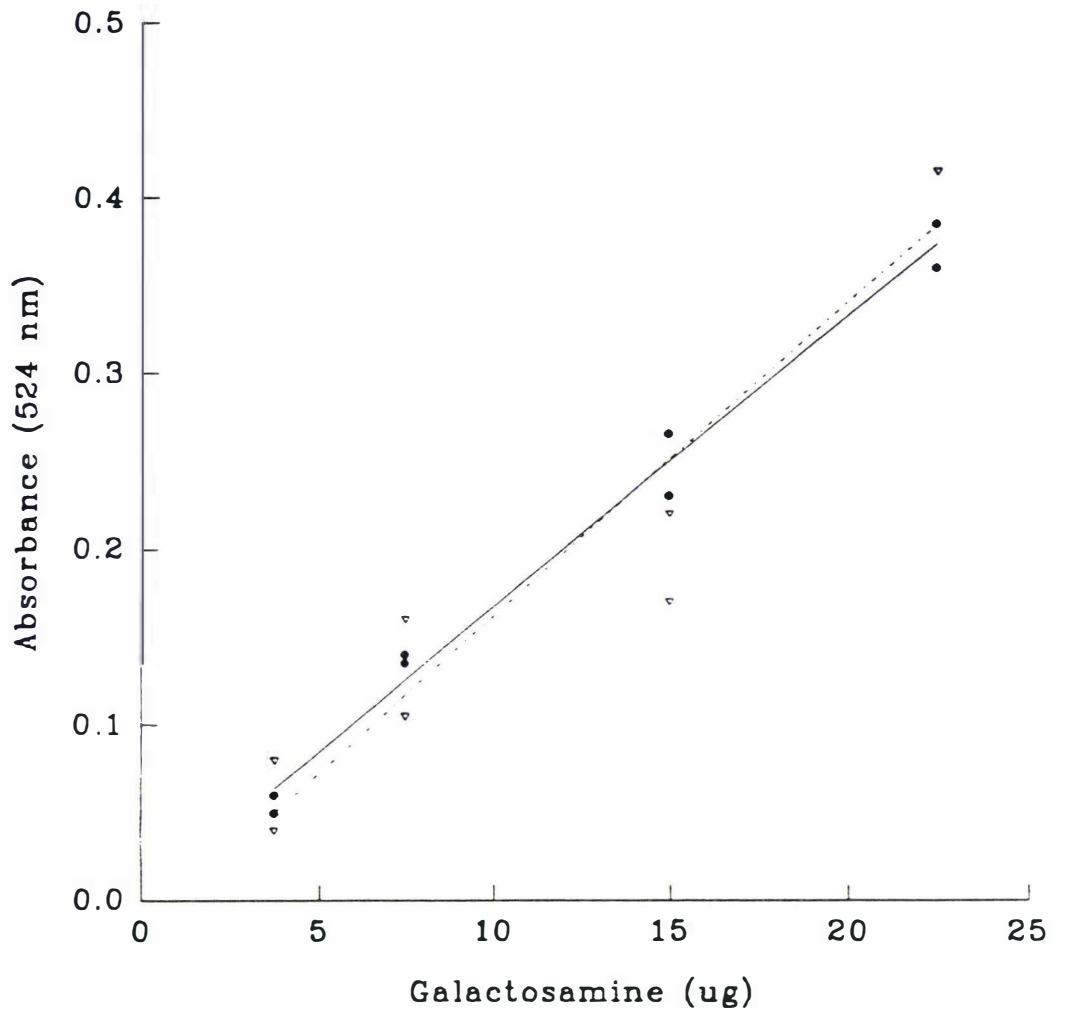


Figure 2.17 Absorption spectra of DMEM and gentamicin following dialysis and oven digestion in 2N HCl (relative to a H<sub>2</sub>O blank, A = 2.0)



**Figure 2.18 Comparison of chondroitin sulphate standards dissolved in water and DMEM after dialysis and oven digestion.**

- Chondroitin sulphate standards in water (solid regression line).
- ▽ Chondroitin sulphate standards in DMEM (dashed regression line).

### 3. Separation of chondroitin sulphate from solutions containing serum proteins

Dialysis could be expected to remove most of the interference in the assay of amino sugars from DMEM and drugs with relatively small molecular weights. However, the molecular weights of equine serum proteins are much too large to pass through any dialysis membrane which would still retain both glycosaminoglycan and proteoglycan molecules. Other processing techniques were thus needed to separate glycosaminoglycan and proteoglycan molecules from equine serum proteins.

#### Experiment 1.

The use of papain to hydrolyse the serum proteins into smaller peptide fragments was investigated as a means for reducing the interference equine serum had on the assay of amino sugars.

#### Materials and methods

1. A papain solution (50-150 units mL<sup>-1</sup> activity) was prepared by dissolving 500 mg of papain<sup>22</sup>, 100 mg of disodium EDTA, and 10 mg of cysteine hydrochloride in water to a final volume of 10 mL.
2. An aqueous solution of equine serum (15%) was divided into 1 mL aliquots to which 0.1-0.5 mL amounts of the papain solution were added. Papain blanks were prepared by adding 0.2 mL of the papain solution to 1 mL volumes of deionised water. Both sets of solutions were incubated at 65°C for 12 hours.
3. Following incubation, 1 mL amounts were transferred into 5 mL pyrex vials containing 0.5 mL 6N HCl, which were sealed and kept at 100°C for 12 hours, then 0.6 mL amounts were assayed in duplicate for amino sugar content.

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<sup>22</sup> Crude papain, 1-3 units mg<sup>-1</sup> activity, Sigma Chemical Company U.S.A.

## Results and Discussion

Treatment with papain did not prevent the formation of a black precipitate in any of the equine serum supplemented solutions, and increased amounts of papain may be needed to hydrolyse this amount of protein into small enough fragments to prevent the formation of a black precipitate. However, the assay of the papain blanks resulted in a much higher absorbance at 524 nm than any of the amino sugar standards previously assayed (Figure 2.19), and the use of greater amounts of papain to further hydrolyse the serum proteins would be expected to produce even greater interference in the assay of the amino sugar content of proteoglycans.

The use of papain in combination with the Gatt & Berman (1966) method for assaying amino sugars has previously been reported (Richardson & Clark 1990) with no mention of an interfering chromophore being formed. The papain source used in this experiment was not highly purified and it is possible that the chromophore resulted from impurities rather than the papain itself.

### Experiment 2.

The use of a more refined papain formulation was investigated to determine whether the papain or a contaminant was responsible for forming the interfering chromophore.

#### Materials and methods

1. An aqueous solution of equine serum (15%) was divided into 18 x 1 mL aliquots to which 0.05-0.5 mL amounts of a refined papain solution<sup>23</sup> were added, and the solutions incubated at 65°C for 12 hours. Each solution then had 1 mL transferred to a 5 mL pyrex vial containing 0.5 mL 6N HCl, which was sealed and kept at 100°C for 12 hours, before 0.6 mL amounts were assayed in duplicate for amino sugar content.

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<sup>23</sup> Stabilised liquid papain, 100 units ml<sup>-1</sup> activity, Salmond Smith Biolab N.Z. Ltd

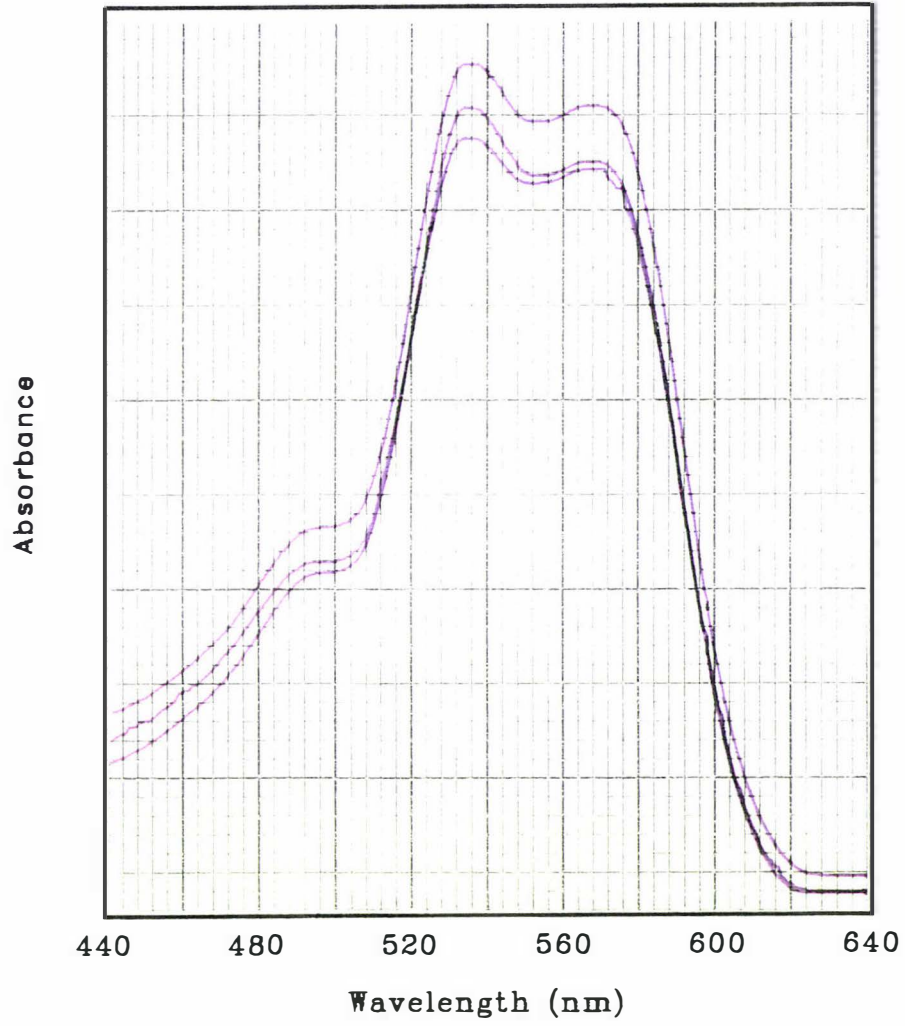


Figure 2.19 Absorption spectrum produced by a crude papain blank solution.

2. From each of the previously prepared glucosamine standards 4 x 0.4 mL amounts were added to 5 mL pyrex vials containing 0.2 mL 6N HCl. To half of these 0.05 mL of the papain solution was added, and to the other half 0.05 mL of water was added. Each vial was sealed, placed in a boiling water bath for 30 minutes, cooled to room temperature, then assayed for amino sugar.

## Results and Discussion

None of the concentrations of papain prevented the formation of a black precipitate in the equine serum supplemented solutions. The absorption spectrum from this papain formulation was similar to that produced by the crude papain solution except the absorption peaks were generally at slightly lower wavelengths (8 to 12 nm), and the peaks corresponding to the previous peaks at 500 and 568 nm were less pronounced (Figure 2.20). The absorbance at 524 nm produced by the 0.05 mL amounts of the papain solution was constant and additive to that produced by the glucosamine standards (Figure 2.21) indicating this papain concentration could possibly be used in this assay if the amino sugar concentrations were relatively high in comparison to the amount of protein to be digested, as would be the case in digests of explants of articular cartilage.

## Experiment 3.

The addition of trichloroacetic acid (TCA) is commonly used to precipitate proteins out of solution. The aim of this experiment was to find the lowest concentration of TCA capable of precipitating enough of the proteins out of a 15% solution of equine serum to prevent the formation of the black precipitate during the subsequent acid digest.

## Materials and methods

1. TCA solutions equivalent to 24, 12, 6 and 3% of saturation were prepared and 0.5 mL amounts of each of these were added to microcentrifuge vials containing 1 mL of a 15% aqueous solution of equine serum. These were kept at 4°C for 2 hours, then centrifuged at 1900 g for 5 minutes.

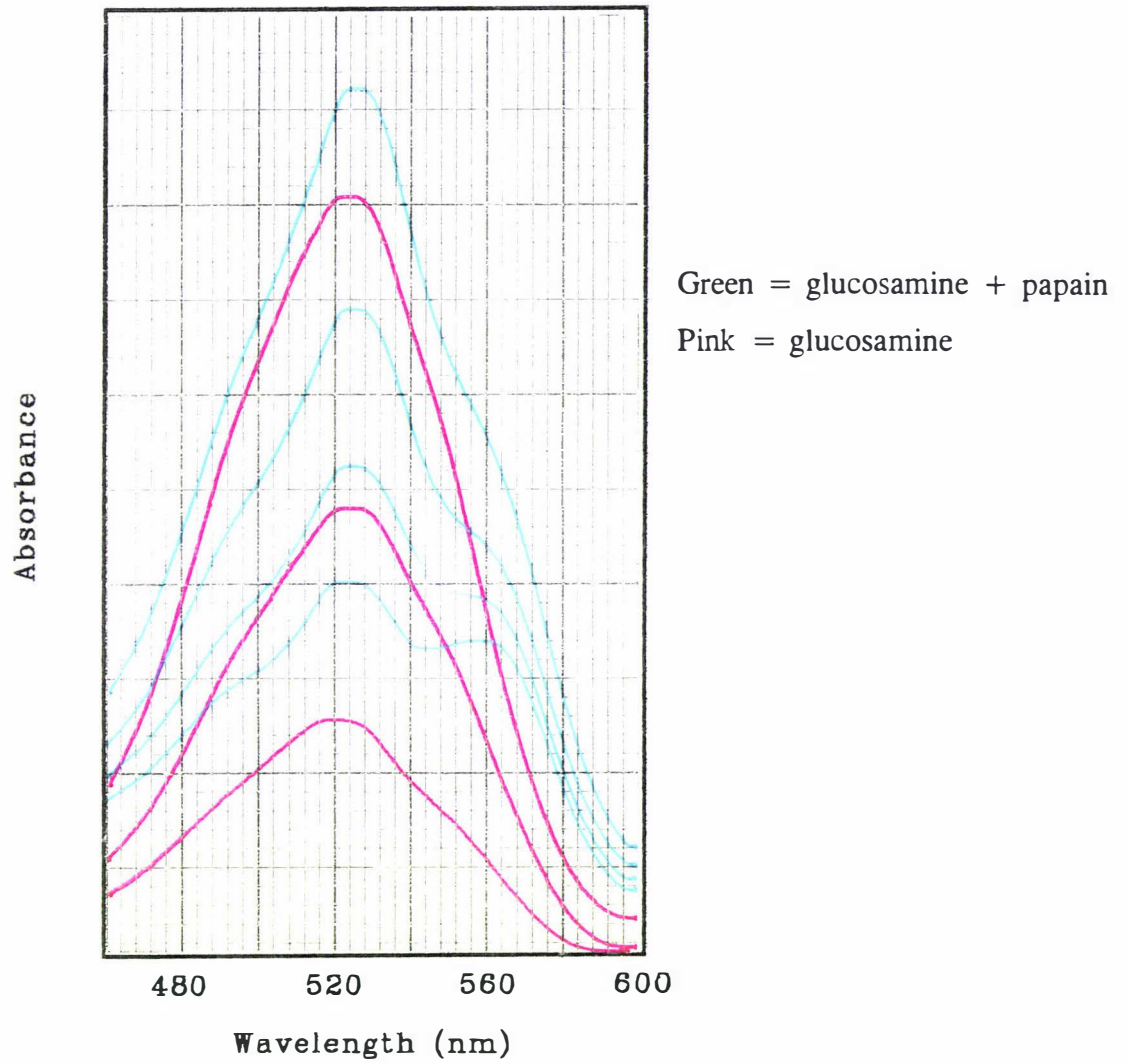


Figure 2.20 Comparison of the absorption spectrum of glucosamine, and a glucosamine + papain combination.

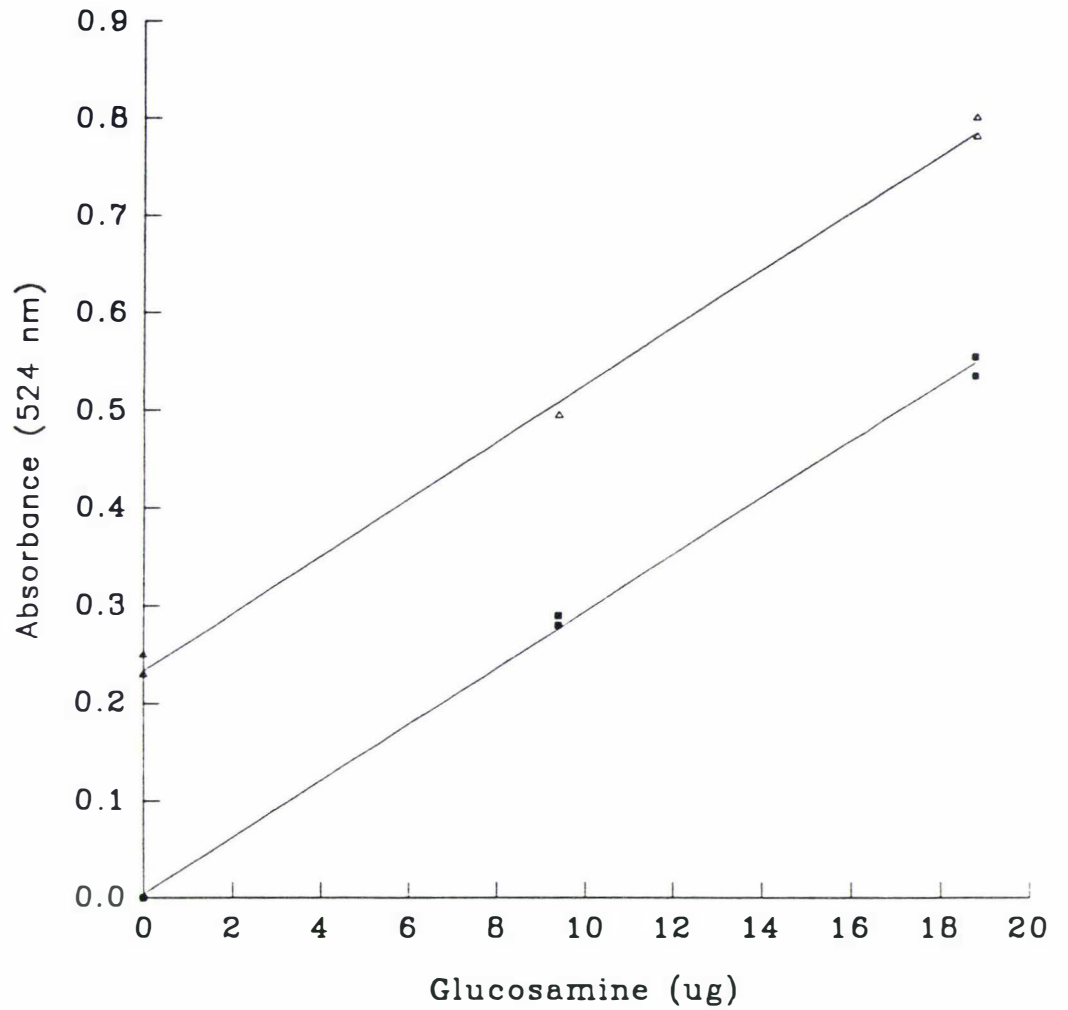


Figure 2.21 Effect of papain on the assay of glucosamine standards.

- Δ Glucosamine + 50 μl papain
- Glucosamine

2. Supernatant (1.2 mL) from each vial was transferred to a pyrex vial containing 0.6 mL 6N HCl, which was then sealed and kept at 100°C for 12 hours.

### Results and Discussion

The 0.5 mL amounts of the 24, 12, 6 and 3% TCA solutions, when added to the 1 mL volumes of the 15% serum solution, equated with final TCA concentrations of 8, 4, 2, and 1%. All concentrations were effective in causing at least some degree of precipitation initially, but only the 2, 4 and 8% final concentrations were completely effective in preventing the formation of a black precipitate during the subsequent 12 hour acid digest at 100°C. Use of TCA to precipitate the equine serum out of solution would require further processing of the supernatants to then remove the TCA itself, before these samples could have their amino sugar content assayed by the method of Gatt & Berman (1966).

### Experiment 4.

The use of TCA precipitation of the serum proteins followed by dialysis of the resulting supernatants against deionised water was investigated as a means of reducing the interference from both equine serum (ES) and DMEM on the estimation of the amino sugar content of chondroitin sulphate standards.

### Materials and methods

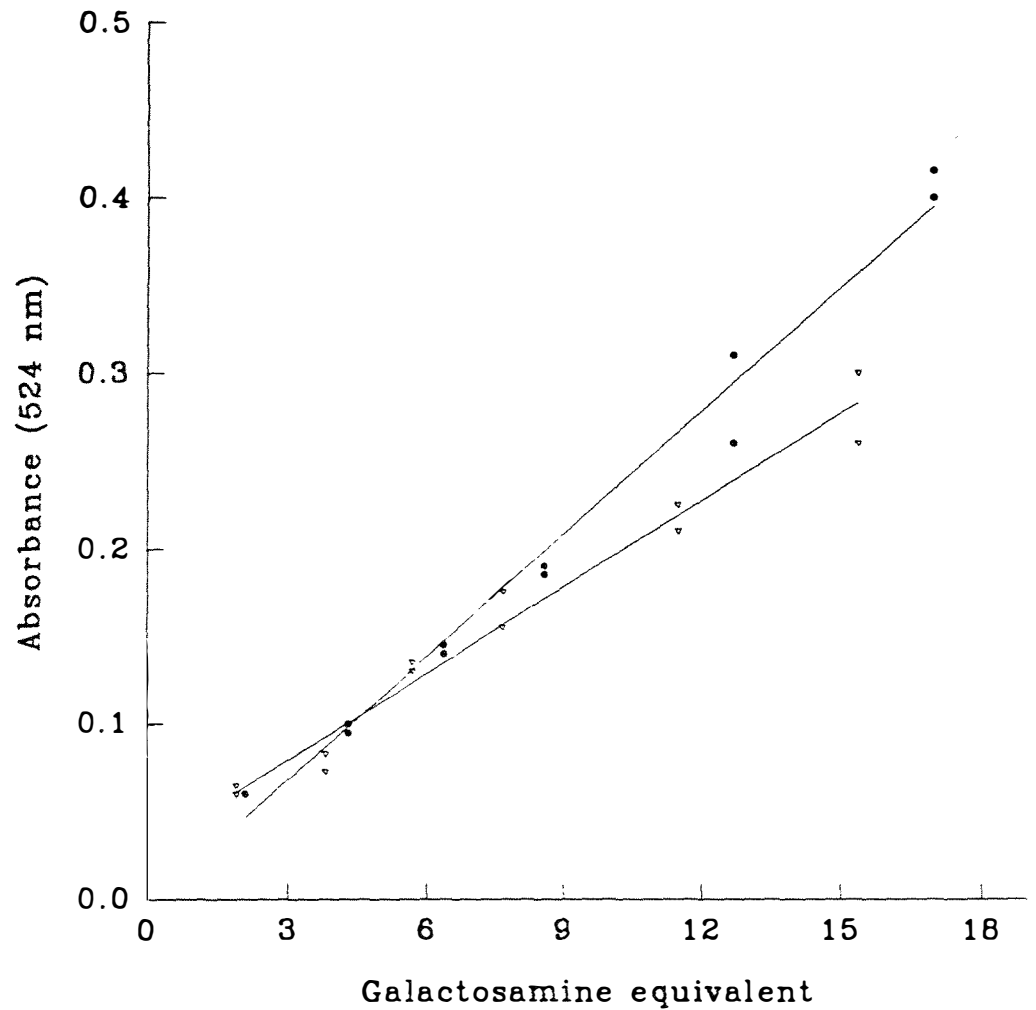
1. ES was added to a portion of the chondroitin sulphate in DMEM stock solution to produce a new chondroitin sulphate in DMEM + 15% ES stock solution. This stock solution was then serially diluted with DMEM+ES to produce standard solutions containing the equivalent of 0-85  $\mu\text{g mL}^{-1}$  galactosamine in DMEM+ES.
2. A second set of water based standards were prepared by dissolving 0.170 g of chondroitin sulphate in a 15% aqueous solution of ES (15% aqES) to a final volume of 100 mL. This stock solution was then serially diluted with 15%

aqES to produce standard solutions containing the equivalent of 0-62  $\mu\text{g mL}^{-1}$  galactosamine in 15% aqES.

3. From these standard solutions 1 mL amounts were transferred to microcentrifuge vials containing 0.5 mL of 12% TCA, which were then kept at 4°C for two hours, before being centrifuged at 1900 g for 5 minutes.
4. The supernatants (1 mL amounts) were dialysed for 36 hours at 4°C against two changes of water, each volume of water being the equivalent of 100 times the combined volume being dialysed.
5. The contents of the dialysis sacks were emptied into calibrated vials, had their volumes standardised with water to 1.5 mL, then 1.4 mL were transferred into pyrex vials containing 0.7 mL 6N HCl. These vials were sealed and kept at 100°C for 12 hours before 0.6 mL amounts were assayed in triplicate.

## Results and Discussion

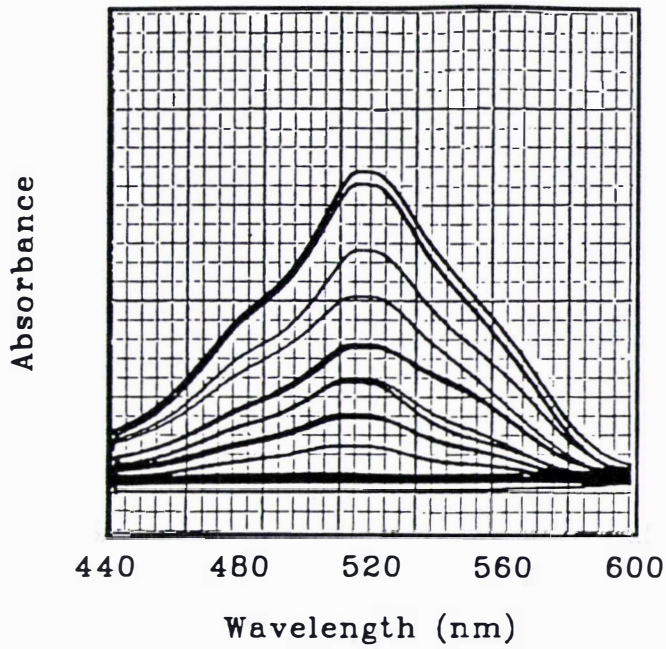
Each 0.6 mL amount finally assayed was equivalent to 0.178 of the original amino sugar concentration of the standard. There was little difference between the chondroitin sulphate in DMEM and the chondroitin sulphate in 15% aqES standard curves (Figure 2.22). Both sets of standards produced similar sized and shaped absorption spectra to the previously assayed chondroitin sulphate standards (Figures 2.23 & 2.24). Protein precipitation by TCA, followed by dialysis, was effective in removing most of the interference associated with the serum supplemented culture medium from the assay of the amino sugar content of chondroitin sulphate standards. The small associated reduction in amino sugar yield is within acceptable limits and appears proportional to the concentration of the chondroitin sulphate being assayed. Chondroitin sulphate standards were used in this experiment because proteoglycan standards were not readily available commercially. Although this method appeared acceptable for assaying chondroitin sulphate from serum supplemented culture medium it was not known if the much larger and complex proteoglycan molecules would behave in a similar fashion.



**Figure 2.22** Effect of serum protein precipitation on the amino sugar concentration of chondroitin sulphate standards.

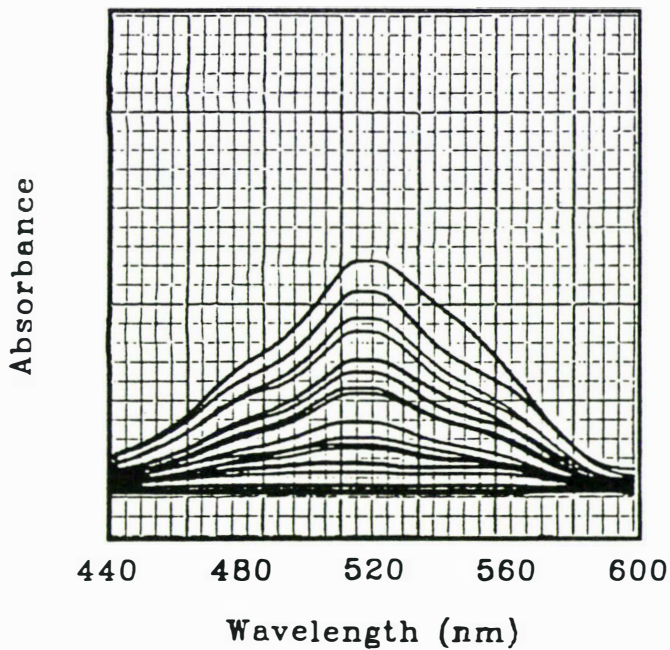
Equine serum (ES) supplemented chondroitin sulphate standards dissolved in either water or DMEM had their protein component precipitated with trichloroacetic acid before being dialysed, oven digested in 2N HCl, and assayed for their amino sugar content.

- Chondroitin sulphate in water + ES
- ▽ Chondroitin sulphate in DMEM + ES



Equivalent concentration of galactosamine of each pair of absorption spectra: 17.0, 12.7, 8.6, 6.4, 4.3, 2.1  $\mu\text{g}$ .

**Figure 2.23** Effect of serum protein precipitation and dialysis on the absorption spectra of aqueous chondroitin sulphate standards.



Equivalent concentration of galactosamine of each pair of absorption spectra: 15.4, 11.5, 7.7, 5.7, 3.8, 1.9  $\mu\text{g}$ .

**Figure 2.24** Effect of serum protein precipitation and dialysis on the absorption spectra of chondroitin sulphate standards in DMEM.

#### 4. Separation of proteoglycans from solutions containing serum proteins

##### Experiment 1.

The aims of this experiment were to determine if either 2 or 4% concentrations of TCA resulted in the precipitation of proteoglycans from solutions (a) containing ES, or (b) not containing ES.

##### Materials and methods

1. Strips of articular cartilage were harvested from the intercarpal joints of a freshly euthanased 8 month old thoroughbred filly and incubated at 37°C in sterile water for 48 hours (approximately 6 mg of cartilage mL<sup>-1</sup> water).
2. Portions of the resulting crude proteoglycan solution had water or serum added, corresponding to 15% of the final volume of each solution, to form a proteoglycan (PG) stock, and a proteoglycan + serum (PG+ES) stock.
3. From the PG stock solution 18 x 1 mL aliquots were transferred into three groups of six microcentrifuge vials. To the first group 0.2 mL of water was added, to the second group 0.2 mL of 12% TCA was added, and to the third group 0.2 mL of 24% TCA was added, resulting in solutions with final TCA concentrations of 0, 2, and 4% respectively.
4. From the PG+ES stock solution 12 x 1 mL aliquots were transferred into two groups of six microcentrifuge vials. To the first group 0.2 mL of 12% TCA was added, and to the second 0.2 mL of 24% TCA was added
5. Each vial was kept at 4°C for 2 hours, centrifuged at 15000 g for 5 minutes, then 1 mL of supernatant transferred into a dialysis sack and dialysed for 24 hours against two changes of 100 equivalent volumes of chilled deionised water.
6. The contents of the dialysis sacks were emptied into calibrated vials, had their volumes standardised with water to 1.5 mL, then 1.4 mL were transferred into pyrex vials containing 0.7 mL 6N HCl. The vials were sealed and kept at 100°C for 12 hours before 0.6 mL amounts were assayed in triplicate for amino sugar content.

## Results and Discussion

The average absorbances of the samples from the PG stock solution treated with 2 and 4% TCA were similar to that of the PG control group. However, the average absorbances of the samples from the PG + ES stock solution treated with either 2 or 4% TCA were markedly different from that of the control group (Table 2.3).

The results indicated that TCA does not directly precipitate proteoglycans from aqueous solutions, but that the use of TCA to precipitate ES proteins can result in a proportion of the proteoglycan molecules being coprecipitated. Extreme pH changes, caused by strong acids such as TCA, cause denaturation of proteins resulting in the formation of random coil structures. In solution these random coils become entangled with each other causing large aggregates and ultimately resulting in precipitation. It is likely some of the proteoglycan molecules also became entangled in these large precipitated aggregates. However, neither the factors influencing the extent of proteoglycan coprecipitation, nor the characteristics of the proteoglycan solutions to be assayed are known. The continued use of TCA to precipitate serum proteins was thus likely to result in variable and unpredictable amino sugar assay results.

<b>Solution</b>	<b>n</b>	<b>Mean Absorbance</b>	<b>SD</b>
<b>PG Control</b>	<b>6</b>	<b>0.105</b>	<b>0.006</b>
<b>PG + 2% TCA</b>	<b>6</b>	<b>0.102</b>	<b>0.002</b>
<b>PG + 4% TCA</b>	<b>6</b>	<b>0.106</b>	<b>0.004</b>
<b>PG+ES + 2% TCA</b>	<b>6</b>	<b>0.061</b>	<b>0.006</b>
<b>PG+ES + 4% TCA</b>	<b>6</b>	<b>0.051</b>	<b>0.002</b>

**Table 2.3** Amino sugar assay absorbances following the addition of TCA and dialysis (524 nm).

## Experiment 2.

Because of the coprecipitation problems associated with the use of TCA other precipitation methods were investigated. Precipitation by increasing the ionic strength of a solution is known as salting out. Salting out is dependant on the existence of groups with hydrophobic characteristics on the surface of proteins. When the protein is in solution the water molecules are forced into close contact with the hydrophobic groups, isolating them from attracting other similar groups. When salts are added to the solution water molecules solvate these ions and as the salt concentration increases water molecules are increasingly drawn away from the hydrophobic groups on the surface of the protein molecules leaving them free to aggregate with the hydrophobic groups on other protein molecules.

Proteoglycans are highly charged molecules consisting of approximately 10% protein and 90% glycosaminoglycans, with the glycosaminoglycans contributing most of the charged groups. The protein component forms the central core of the molecule from which the glycosaminoglycan side chains radiate. The solubility of the proteoglycan molecules would thus be less likely to be affected by increasing salt concentrations than would the solubility of the serum proteins.

The aim of this experiment was to investigate whether the use of ammonium sulphate to precipitate the serum protein component of the solutions would affect the proteoglycan concentration of the solution or the assay of its amino sugar component.

### Materials and methods

1. A proteoglycan (PG) stock solution and a proteoglycan stock solution with 15% added serum (PG+ES) were prepared in a similar fashion to those prepared in experiment one.
2. Saturated ammonium sulphate solution (0.5 mL) was added to six microcentrifuge vials containing 1 mL of the PG stock solution and to six vials containing 1 mL of the PG+ES stock solution. Water (0.5 mL) was added to

an additional twelve vials which contained just 1 mL of the PG stock solution.

3. All vials were left at room temperature for 4 hours before being centrifuged at 15000 g for 5 minutes. Six of the twelve vials containing PG and water had 1 mL removed and stored at 4°C. All of the other vials had 1 mL transferred into dialysis sacks, which were dialysed for 24 hours against two changes of 100 volumes of chilled (4°C) deionised water.
4. The contents of the dialysis sacks, and the stored PG + water samples, were emptied into calibrated vials, had their volumes standardised with water to 2.0 mL, then 1.8 mL were transferred into pyrex vials containing 0.9 mL 6N HCl. These vials were sealed and kept at 100°C for 12 hours, before 0.6 mL amounts were assayed in triplicate for amino sugar content.

## Results and Discussion

The high osmotic potential created by the addition of the ammonium sulphate resulted in a net increase in the volume within each dialysis sack. Ammonium sulphate in combination with dialysis was effective in preventing the development of a black precipitate during the subsequent acid digestion. Dialysis did not greatly affect the yield of amino sugars from the PG solutions. The addition of ammonium sulphate to the PG solutions prior to dialysis made no difference to the absorbances recorded from the assay, although the final amino sugar concentration of the proteoglycan solution subjected to these procedures was close to the lowest sensitivity of the assay. However, the addition of ammonium sulphate to the PG+ES solutions resulted in mean absorbances approximately 10 fold higher than the other three groups (Table 2.4).

<b>Solution</b>	<b>n</b>	<b>Mean</b>	<b>SD</b>
<b>Non-dialysed PG Control</b>	6	0.054	0.003
<b>Dialysed PG Control</b>	6	0.050	0.005
<b>PG + NH<sub>4</sub>SO<sub>4</sub></b>	6	0.056	0.004
<b>PG+ES + NH<sub>4</sub>SO<sub>4</sub></b>	6	0.620	0.029

**Table 2.4 Amino sugar assay absorbances (524 nm) following the addition of NH<sub>4</sub>SO<sub>4</sub> and dialysis.**

## 5. Summary

The concentrations of papain used were insufficient to hydrolyse enough of the serum proteins to prevent the formation of a black precipitate on subsequent acid digestion. Furthermore, even the lowest concentrations of papain directly reacted with the assay to produce an absorbance at 524 nm capable of causing interference.

The use of TCA to precipitate the serum proteins caused a coprecipitation of an unpredictable proportion of the proteoglycan content from the solution. Ammonium sulphate precipitated sufficient serum protein from solution to prevent the formation of a black precipitate on subsequent acid digestion. However, its use resulted in absorbances at 524 nm, which were unrelated to the known amino sugar content.

The solubility of a protein molecule in an aqueous solution is determined by the distribution of charged hydrophilic and hydrophobic groups on the surface of the protein. Protein precipitates are formed by the aggregation of protein molecules and can be induced by changes in pH, changes in ionic strength, the addition of organic miscible solvents, or the addition of other inert solutes or polymers. Temperature may also affect the degree of aggregation achieved.

Proteins can be precipitated out of solution by the addition of water miscible organic solvents such as acetone and ethanol. Addition of organic solvent lowers the dielectric

constant of the solution and hence its solvating power. As a consequence aggregation of protein molecules through electrostatic attraction occurs. This occurs more readily when the protein molecules are large and when the pH is close to the isoelectric point of the protein. The use of water miscible solvents was not attempted as the method is also often used to precipitate proteoglycans out of solutions.

Precipitation of a protein from a solution can also be induced by adjusting the pH of the solution to close to the isoelectric point of that protein. At the isoelectric point the number of negatively charged groups equals the number of positively charged groups and aggregation of protein molecules occurs due to electrostatic attractions. Culture medium contains buffers against changes in acidity. The buffering capacity is altered by the surrounding gaseous environment, the amount and type of metabolic products produced by the cultured tissue, and the concentration and type of drug added to the culture medium. Precipitating the serum proteins by adjusting the pH to the isoelectric point of serum albumin was also considered not feasible as it would have involved having to titrate each and every culture medium sample to a defined pH.

A variety of chromatographic techniques were investigated. However, equipment availability, cost, and technical problems, associated with processing several hundred samples of small volume and very low concentration without diluting each sample below the minimum sensitivity of the amino sugar assay precluded use of most of these techniques.

Serum was required in the culture medium for several reasons. Serum has been shown to contain a number of 'factors' important for the maintenance of tissue health and metabolic activity during culture. In some media the use of serum has been replaced with the addition of known quantities of albumin and growth factors, but this was not an option in the current work because the above experiments suggested albumin was a major component of the precipitate that formed. Synovial fluid is a highly viscous dialysate of plasma consisting of approximately 25 mg of protein mL<sup>-1</sup> (Korenek *et al.*, 1992). If the model was to represent the effects of synovial fluid concentrations of

certain drugs on articular cartilage, then a significant protein concentration was required in the culture medium.

The measurement of proteoglycans released into the culture medium was dropped from the proposed experimental protocol at this point. Many complex sample manipulations would have been required to remove media, serum, and drug interference from the assay of the amino sugar content of the proteoglycans. Not only would these procedures have been costly with respect to the time and resources required, but the size of the combined experimental errors associated with the number of sample manipulations would have also greatly reduced the value of the results.

## C. EXPLANT CULTURE CHARACTERISTICS

### 1. Variation in the amino sugar content and rate of $^{35}\text{S}$ incorporation between explants from different sites

The number of samples needed to demonstrate a difference between two groups depends on both the size of the difference and the variance of the population. Because only small differences between treatment groups were expected the variances associated with the initial amino sugar and cellular contents of the explants needed to be as small as possible for sample numbers to be kept within manageable limits. The variability of the amino sugar content, and rate of  $^{35}\text{S}$  incorporation, within and between sites in the middle carpal joints of a single horse were thus determined.

#### Materials and methods

1. Two hours after the barbiturate euthanasia of an 18 month old thoroughbred colt, both carpi were skinned and disarticulated at the middle carpal joint. The cartilage chisel was used to harvest 4 mm wide full thickness strips of articular cartilage from the left and right middle carpal joint surface of the radial, third, and intermediate carpal bones. The cartilage strips from these six sites were placed in separate screw-capped jars containing chilled ( $4^{\circ}\text{C}$ ) DMEM (pH 7.4) and PSK.
2. Ten explants of articular cartilage (3 mm diameter) were cut from each of these six sites, and were individually blotted dry, weighed, and placed in 0.5 mL of DMEM+ES (pH 7.4). All explants were incubated at  $37^{\circ}\text{C}$  in a humidified atmosphere consisting of 5% carbon dioxide and air, with the culture medium changed and replaced with DMEM+ES supplemented with  $5\ \mu\text{Ci mL}^{-1}\ \text{Na}_2^{35}\text{SO}_4$  after 48 hours. Each explant was cultured for a further 24 hours before being subjected to the three wash procedure as described earlier.
3. Each explant was placed in a sealed pyrex vial containing 5 mL 2N HCl, kept at  $100^{\circ}\text{C}$  for 12 hours, then duplicate 0.6 mL amounts were assayed for amino sugar content, and 1 mL amounts were mixed with emulsion scintillation

mixture in duplicate and placed in a scintillation counter. Amino sugar  $\text{mg}^{-1}$  wet weight and  $\text{dpm mg}^{-1}$  wet weight values for each explant were then calculated by the methods previously described.

## Results and Discussion

For each explant, the amino sugar assay was used as a measure of the proteoglycan content, while the incorporation of  $^{35}\text{S}$ , as reflected by the calculated  $\text{dpm}$  values, was used as a relative measure of both cellular content and synthetic activity. The between explant variability in amino sugar content (Table 2.5) and incorporation of  $^{35}\text{S}$  (Table 2.6) was least in the group of explants from the third carpal bones. However, relatively large within site variation existed in all of the sites. Generally, sites which had a large range in explant weights also tended to have more variable results, implying standardising by weight was not accounting for much of the between explant variation. Possible reasons for this included; (a) inaccuracies in weight determination due to variations between explants in surface wetness at weighing, (b) differences in explant composition caused by variations in sampling depths between explants, and (c) inherent within site variations in amino sugar content and cellularity.

The results indicate that ideally the use of explants from a single site is preferable, but where greater explant numbers are needed the use of the same site in both legs may still be acceptable. Care must be taken to ensure explants are of a similar dryness, weight, and sample depth if the variation between explants is to be kept to a minimum. The use of a DNA assay to standardise each explant may further reduce the variability between explants due to differences in cellularity.

Carpal bone	Side	Explants (n)	Weight $\pm$ SD (mg)	CV (%)	AS $\pm$ SD ( $\mu\text{g mg}^{-1}$ )	CV (%)
Radial	L	9	4.64 $\pm$ 0.77	17	13.30 $\pm$ 2.61	20
Radial	R	10	4.60 $\pm$ 1.13	25	12.02 $\pm$ 3.98	33
Third	L	10	5.69 $\pm$ 0.35	6	13.52 $\pm$ 2.09	16
Third	R	10	4.83 $\pm$ 0.63	13	14.84 $\pm$ 1.96	13
Intermediate	L	10	5.36 $\pm$ 0.78	15	12.73 $\pm$ 4.55	36
Intermediate	R	10	5.62 $\pm$ 1.18	21	14.23 $\pm$ 6.04	42

**Table 2.5** Variability in the amino sugar content (AS) of cultured explants from different carpal bones ( $\mu\text{g mg}^{-1}$ )

Carpal bone	Side	Explants (n)	Weight $\pm$ SD (mg)	CV (%)	$^{35}\text{S}$ $\pm$ SD (dpm $\text{mg}^{-1}$ )	CV (%)
Radial	L	9	4.64 $\pm$ 0.77	17	16440 $\pm$ 4125	25
Radial	R	10	4.60 $\pm$ 1.13	25	15790 $\pm$ 6890	43
Third	L	10	5.69 $\pm$ 0.35	6	14490 $\pm$ 3475	24
Third	R	10	4.83 $\pm$ 0.63	13	11275 $\pm$ 2000	17
Intermediate	L	10	5.36 $\pm$ 0.78	15	14215 $\pm$ 2790	19
Intermediate	R	10	5.62 $\pm$ 1.18	21	13710 $\pm$ 4605	33

**Table 2.6** Variability in the incorporation of  $^{35}\text{S}$  by cultured explants from different carpal bones (dpm  $\text{mg}^{-1}$ )

## 2. Viability of chondrocytes in cultured explants of equine articular cartilage

The aim of this experiment was to determine the effect of tissue culture duration on the viability of chondrocytes in explants of equine carpal articular cartilage.

### Materials and methods

1. Two hours after the barbiturate euthanasia of a five year old thoroughbred gelding, both carpi were skinned and disarticulated at the middle carpal joint. A razor blade was used to harvest full thickness strips of articular cartilage from the exposed carpal bones and the strips were placed in a screw-capped jar containing chilled (4 °C) DMEM (pH 7.4) and PSK. A cartilage punch was then used to cut 30 x 3 mm explants from these strips, and each explant was randomly allocated to one of six groups until each group contained five explants.
2. The first group of five explants was immediately fixed in glutaraldehyde (2.5% concentration in phosphate buffer at pH 7.2) for 6 hours, rinsed in three changes of PBS, and stored in 70% ethanol. The remaining 25 explants were placed in individual tissue culture wells containing 1.0 mL of DMEM+ES (supplemented with 100 IU mL<sup>-1</sup> penicillin and 100 µg mL<sup>-1</sup> streptomycin) and were incubated at 37 °C in a humidified atmosphere of 5% carbon dioxide and air.
3. The culture medium for each explant was changed on days 1, 2, 4, 6, and 8. At each change one group of five explants was removed from culture and fixed in glutaraldehyde, rinsed in PBS, and stored in 70% ethanol.
4. Each of the fixed explants was cut in half, and one half was processed and embedded in paraffin wax while the other half was stored. Sections (6µ) across the full diameter of each disc were cut and stained with haematoxylin, eosin, and alcian blue (pH 7.2).
5. The total number of nucleated cells in each section was counted at 400 x magnification, followed by a count of the number of dead cells under an oil immersion lens at 1000 x magnification. Cell death was assessed purely on nuclear morphology. Dead cells were defined as 'nucleated cells in which the nuclear material had become pyknotic i.e. the nucleus had shrunk in size and the

chromatin had condensed into a solid dark coloured structureless mass or masses'. Empty lacunae and anuclear cells were not included in either the total or the dead cell counts as neither the orientation of the cell nor the effects of processing could be excluded as factors involved in their appearance.

## Results and Discussion

The results are shown in Table 2.7 and Figure 2.25. Due to difficulties associated with the use of a folded razor blade to harvest strips of cartilage, not all explants were of the same thickness, and the thickness of some explants also varied across their diameter. In addition, because of possible contamination during explant processing 100 IU mL<sup>-1</sup> penicillin and 100 µg mL<sup>-1</sup> streptomycin were used during culture. These concentrations are routinely used during cell culture.

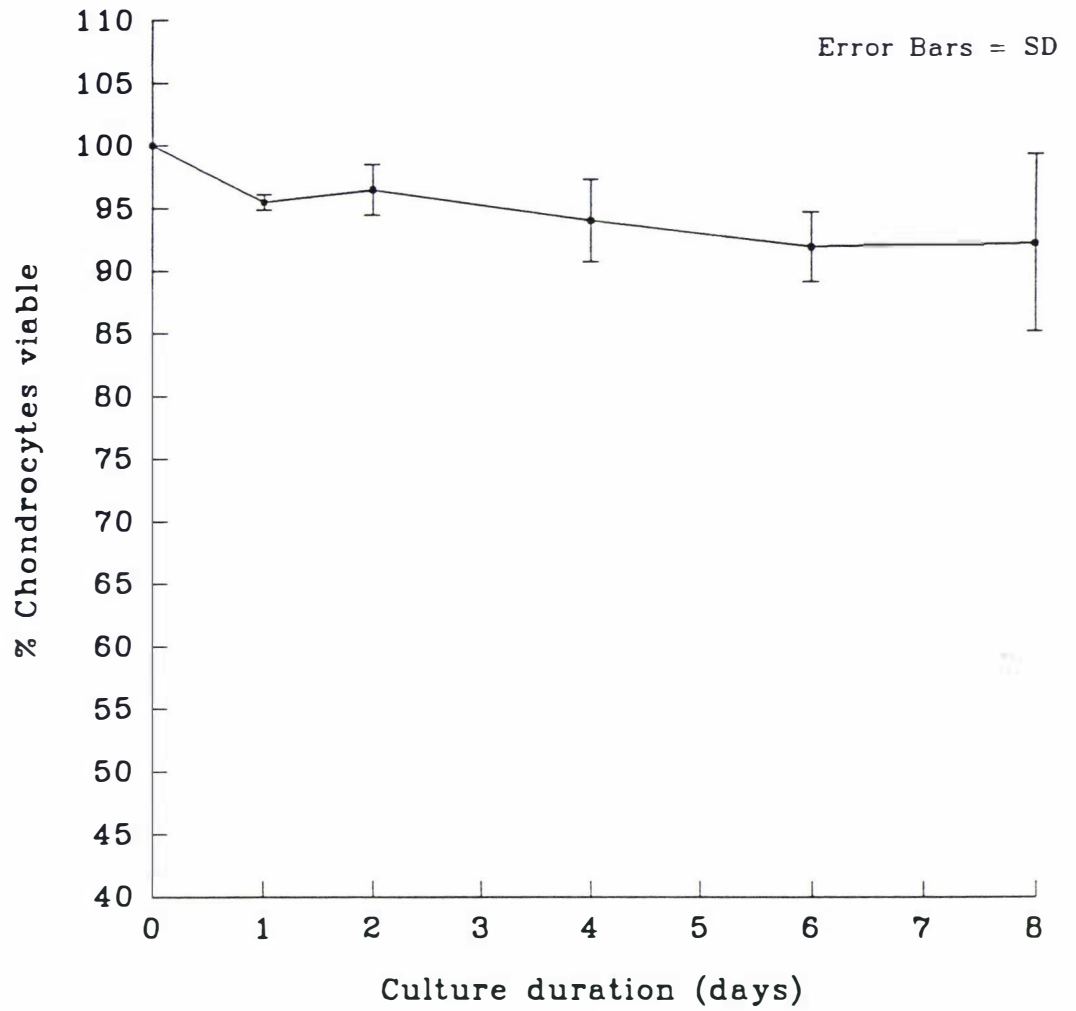
There was an initial increase in the average number of dead cells in the explants after the first day of tissue culture, followed by a more gradual increase over the next 7 days. The average viability of the chondrocytes exceeded 90% for the first 8 days of culture. However, the variability found between sections, generally increased with culture duration. Chondrocyte death was greatest at the ends of each section, where the punch had cut perpendicular to the articular surface (Figure 2.26), followed by chondrocytes in the tangential zone where cell density was greatest (Figure 2.27). Chondrocyte death was least noticeable in the radiate and calcified zones of each section. Cell death at the cut outside edges of the cultured explants was expected. However, the high proportion of cell deaths occurring in the morphologically intact tangential zone in comparison to the radiate and severed calcified zones was not.

Cell health was generally thought to decline during the culture period, as indicated by an increasing prevalence of cytoplasmic vacuolation, anuclear cells, and variable cellular staining characteristics (Figure 2.28). Wide variation in cellular density, cellular distribution characteristics, and width of samples was observed. A more standardised sampling procedure, from a smaller number of morphologically similar sites, may help reduce the variation between explants.

Classification of cell death purely on the presence of a pyknotic nucleus may have underestimated the true percentage of dead chondrocytes, but was considered the most clear-cut and objective assessment. Never-the-less, the study demonstrated that a high percentage of the chondrocytes within tissue cultured explants of equine middle carpal joint articular cartilage can be maintained viable for periods of at least 8 days.

Explant	Non-cultured controls	Days cultured				
		1	2	4	6	8
1	0/465	22/436	19/410	23/559	34/490	69/398
2	0/449	18/467	26/560	34/345	33/450	12/844
3	0/437	16/397	14/758	45/491	74/761	20/461
4	0/442	24/457	23/435	24/515	54/469	70/561
5	0/403	20/440	5/551	9/413	40/563	10/357
<b>Dead</b>	0	100	87	135	138	181
<b>Total</b>	2196	2199	2714	2323	2733	2617
<b>% Dead</b>	0	4.5	3.5	6.0	8.1	7.7
<b>% Viable</b>	100	95.5	96.5	94.0	91.9	92.3
<b>SD</b>	0	0.6	2.0	3.3	2.8	7.0

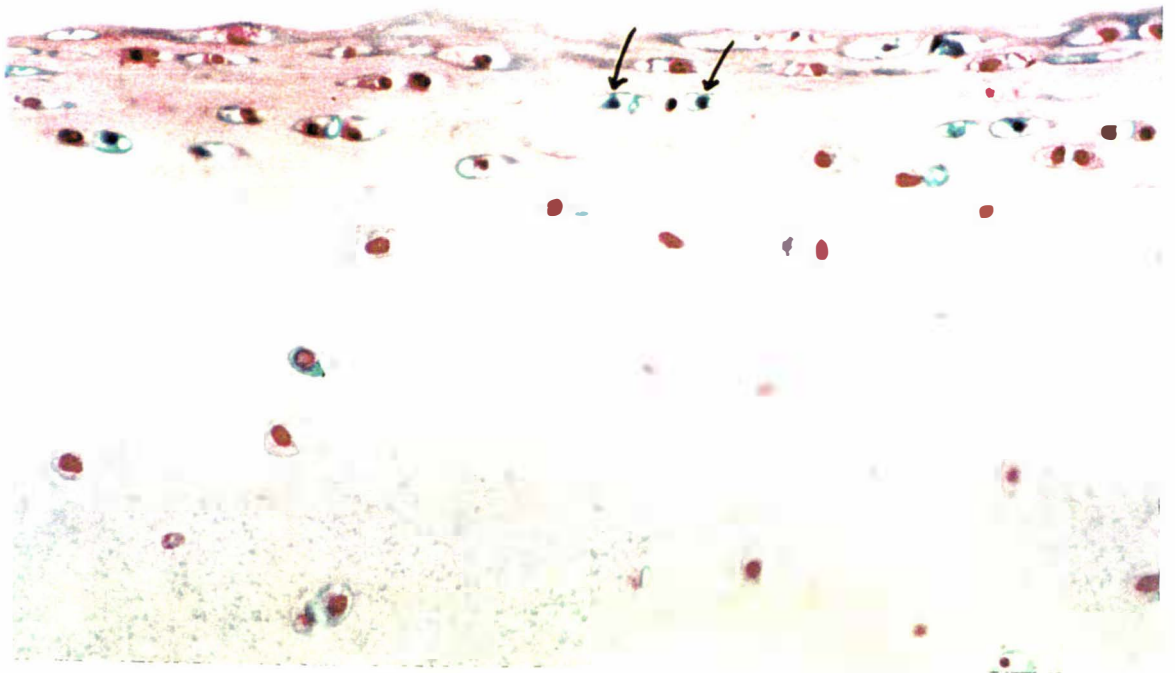
**Table 2.7** Chondrocyte death relative to time cultured (dead/total).



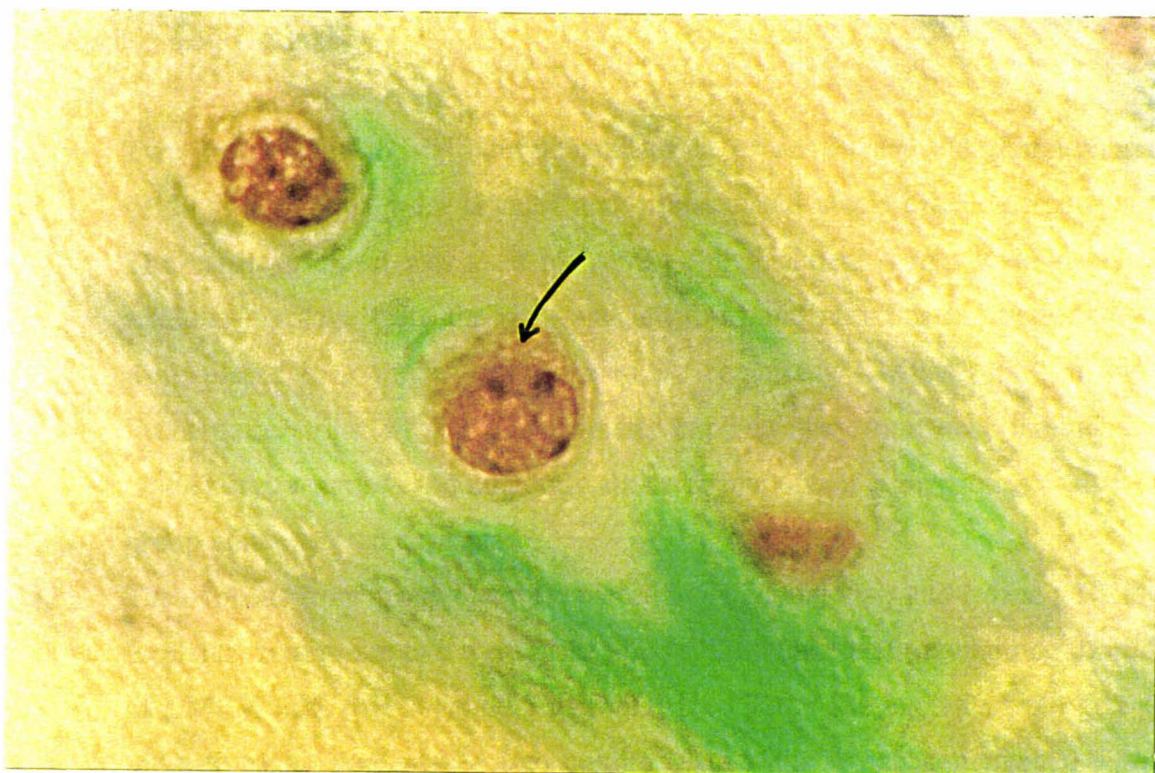
**Figure 2.25** Effect of culture duration on the viability of chondrocytes within explants of equine articular cartilage.



**Figure 2.26** Photomicrograph showing cell death at the edge (↗) of a cultured explant of equine carpal articular cartilage (400x magnification).



**Figure 2.27** Photomicrograph showing cell death at the articular surface (↗) of a cultured explant of equine carpal articular cartilage (1000x magnification).



**Figure 2.28** Photomicrograph showing cell vacuolation (↗) in a cultured explant of equine carpal articular cartilage (4000x magnification).

### 3. Variation in the biosynthetic rate and total amino sugar concentration relative to time cultured

The aims of this experiment were (a) to compare the use of a DNA assay to the use of wet weight as a means for standardising both the amino sugar content and incorporation of  $^{35}\text{S}$  by each explant, and (b) to determine how the amino sugar content and incorporation of  $^{35}\text{S}$  varied with culture duration.

#### Materials and methods

1. Two hours after the barbiturate euthanasia of an 9 year old thoroughbred gelding, both carpi were skinned and disarticulated at the middle carpal joint. The cartilage chisel was used to harvest full thickness 4 mm wide strips of articular cartilage from the right and left third and radial carpal bones which were placed in a single screw-capped jar containing DMEM+PSK at 4°C. From these strips 80 x 3 mm diameter explants were punched, individually blotted dry, weighed, and randomly allocated into one of eight groups until each group contained ten explants.
2. The explants from group one were placed in individual vials and frozen (-20°C). Explants from group two were placed into culture plate wells containing 0.5 mL of DMEM+ES (pH 7.4) and 5  $\mu\text{Ci mL}^{-1}$   $\text{Na}_2^{35}\text{SO}_4$ . The explants from groups three to eight were placed into culture plate wells which contained 0.5 mL DMEM+ES. All groups except group one were then incubated at 37°C in a humidified atmosphere of 5% carbon dioxide and air.
3. After 24 hours of culture the explants from group two were harvested, washed using the previously described three wash procedure, and placed in individual vials and frozen.
4. After 1, 2, 3, and 4 days of culture groups 3, 4, 5, and 6 respectively were placed in radiolabelled culture medium for their final 24 hours of culture before also being harvested, washed and frozen. The culture media of all groups not being radiolabelled were replaced with fresh DMEM+ES every 48 hours.

5. All frozen samples were thawed, had 1 mL of PBS + 50  $\mu$ l of papain<sup>24</sup> added, and were placed in a water bath at 65°C for 24 hours.
6. Calf thymus DNA standards, ranging from 0 to 10  $\mu$ g DNA mL<sup>-1</sup>, were prepared as previously described. From each of the standard solutions, and from each of the papain digested samples, 0.2 mL amounts were transferred into 1 mL polystyrene microcentrifuge tubes, placed in crushed ice and briefly sonicated. The DNA content of 50  $\mu$ l amounts were assayed in duplicate according to the method of Labarca and Paigen (1980) using a 0.1  $\mu$ g mL<sup>-1</sup> concentration of the Hoechst 33258 reagent solution.
6. The remaining 0.8 mL of each of the papain digested samples was mixed with 3.2 mL of 2.5N HCl, three papain blanks were prepared by mixing 50  $\mu$ l of papain with 5 mL of 2N HCl, and a series of galactosamine standards in 2N HCl were also prepared. All vials were sealed and kept at 100°C for 12 hours before 0.6 mL amounts of each were assayed in triplicate for amino sugar content, and 1.0 mL amounts of each had their dpm determined in duplicate, by the previously described method.

## Results and Discussion

The assay of the DNA content of each explant necessitated a number of extra sample manipulations, including the use of a papain digest, which potentially reduced the accuracy of the assay of the amino sugar content of each explant. The concentration of papain in the papain blanks was equivalent to the papain concentration in each sample. The average absorbance (0.084) produced by the assayed papain blanks was subtracted from each of the assayed samples before amino sugar content calculation. There was no apparent advantage gained by using the final DNA content of each explant to standardise its <sup>35</sup>S incorporation and amino sugar content (coefficients of variation = 39 and 26%), compared to the use of the wet weight of each explant (coefficients of variation = 42 & 19%), (Figures 2.29-2.32 & Appendix Tables 8-11). The aim of standardising each explant was to reduce the variation in the initial amino

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<sup>24</sup> Stabilised liquid papain, Salmond Smith Biolab N.Z. Ltd.

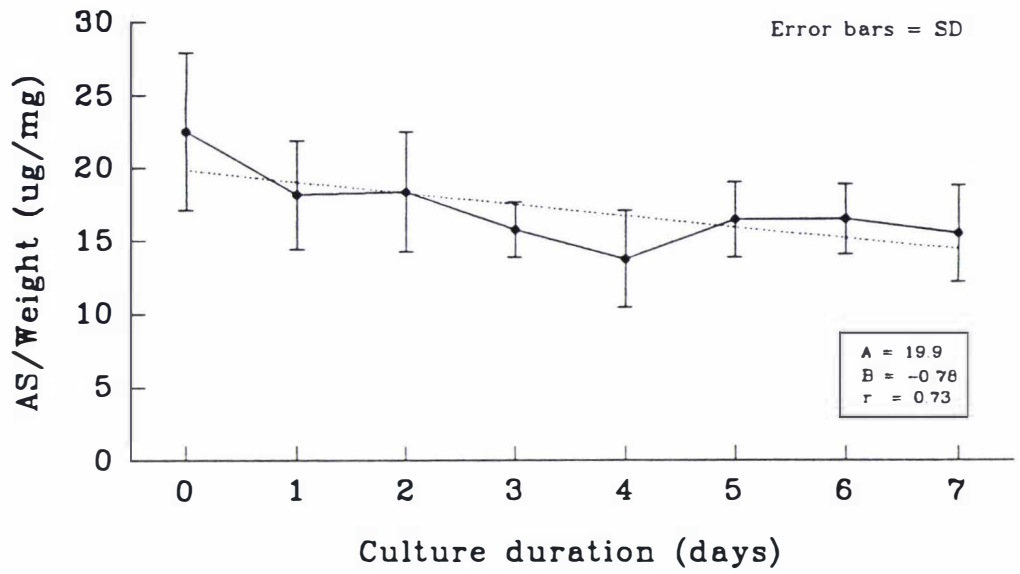


Figure 2.29 Effect of culture duration on amino sugar content of explants relative to their initial wet weight.

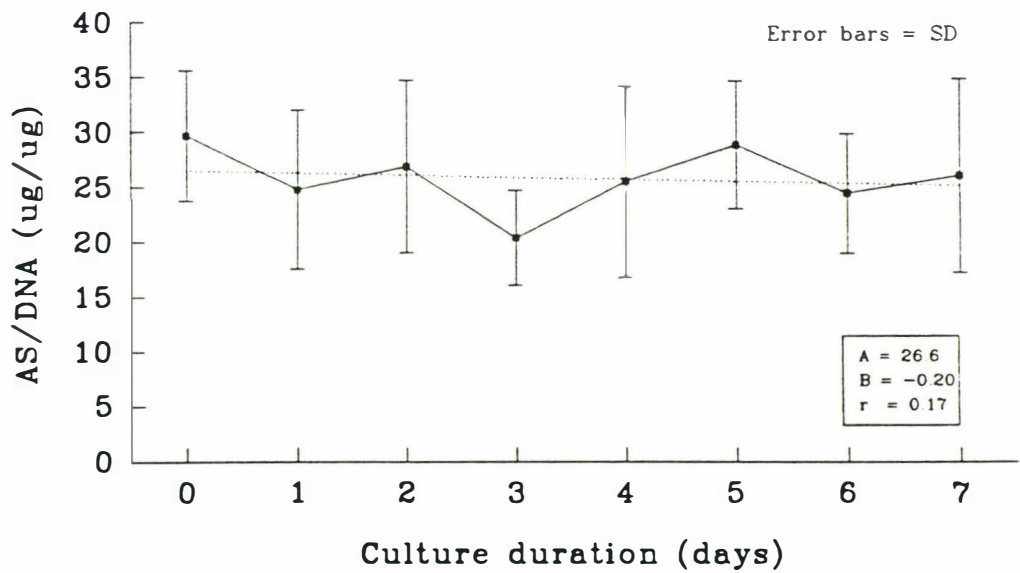


Figure 2.30 Effect of culture duration on amino sugar content of explants relative to their final DNA content.

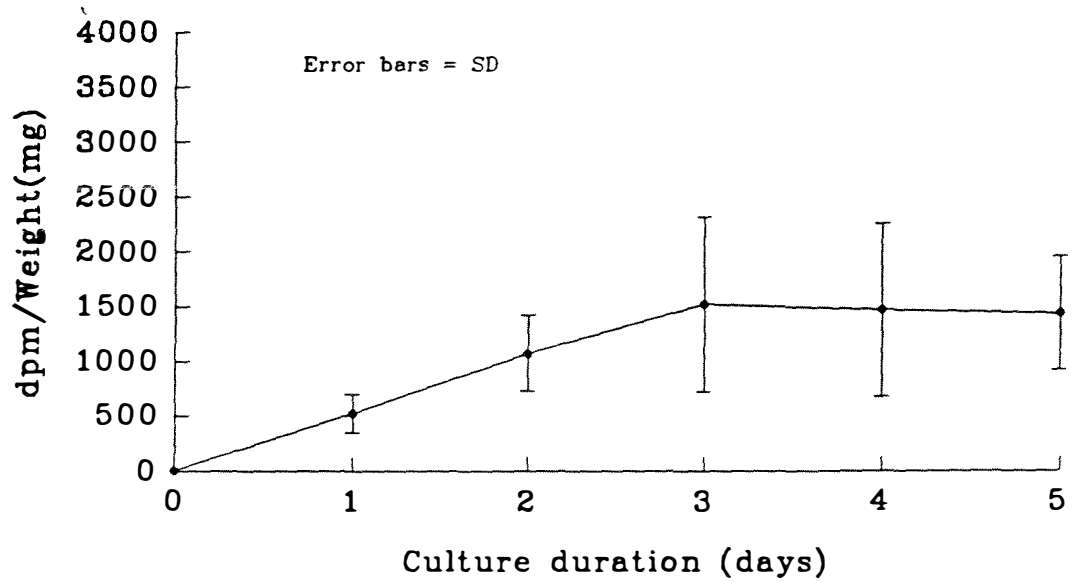


Figure 2.31 Effect of culture duration on <sup>35</sup>S incorporation by explants relative to their initial wet weight.

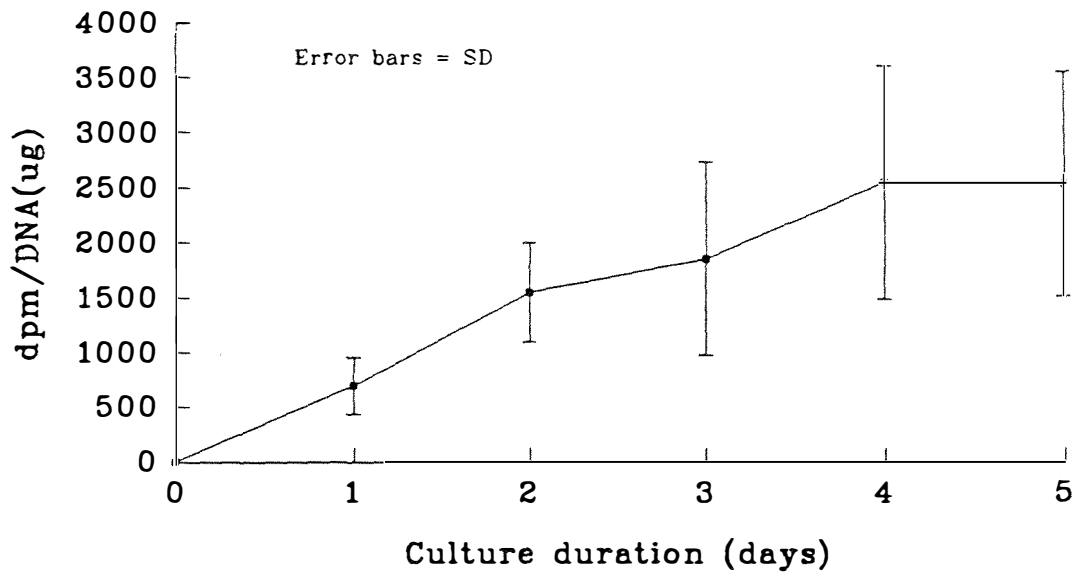


Figure 2.32 Effect of culture duration on <sup>35</sup>S incorporation by explants relative to their final DNA content.

sugar content and cellularity of the explants. How accurately the final DNA content reflects variations in the initial cellular content of the explants is not known. Cytotoxic effects associated with culture and different drug concentrations could also be expected to have variable effects on the final DNA content of each explant. For these reasons, and continuing problems with repeatability, the assay of DNA content was excluded from the proposed experimental protocol.

The amino sugar/weight results indicate cultured explants of equine carpal articular cartilage have a net loss of proteoglycans during tissue culture, with the final proteoglycan content tending to be inversely related to culture duration. In contrast the average rate of proteoglycan synthesis initially increased up to the end of the third day of culture, then remained relatively constant for a period. Delaying both the addition of the drugs to be tested and the initiation of radiolabelling until after the explants had acclimatised to the *in vitro* conditions would thus be more likely to expose concentration related drug effects on proteoglycan synthesis.

## MATERIALS AND METHODS: SECTION TWO

### *USE OF THE MODEL*

#### A. **EXPLANT CULTURE**

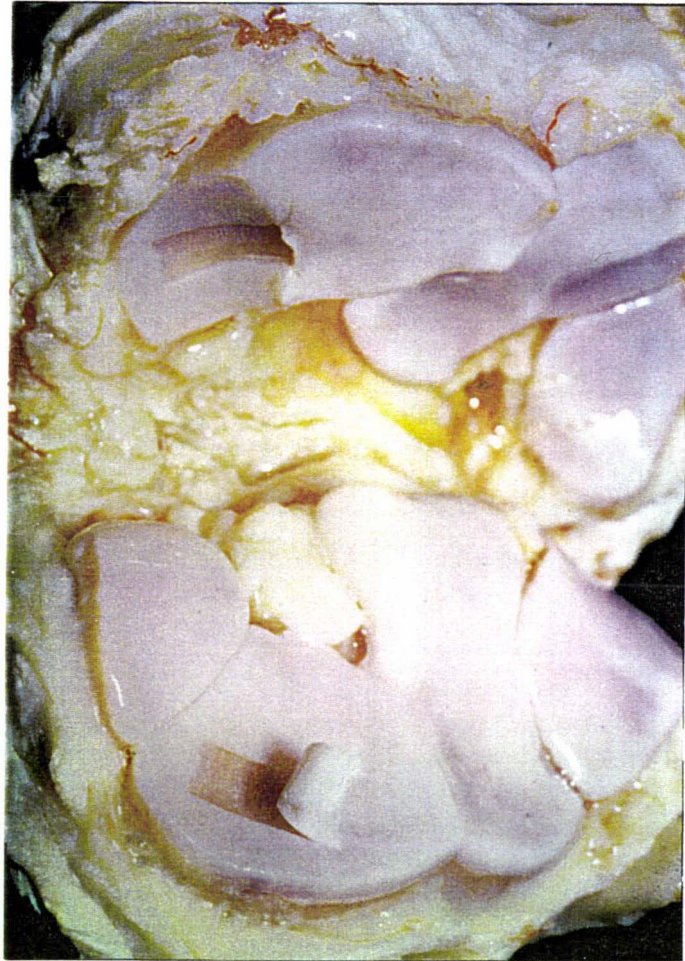
##### 1. **Source of articular cartilage**

Articular cartilage was harvested from the middle carpal joints of 12 thoroughbred or thoroughbred cross horses. These ranged in age from 2 to 12 years and (a) had been presented at the Massey University Equine Clinic and euthanased by barbiturate overdose for reasons unrelated to, and unlikely to affect, their carpal articular cartilages, or (b) were horses from a local hunt club or abattoir which had been killed by a bullet or captive bolt penetrating the brain.

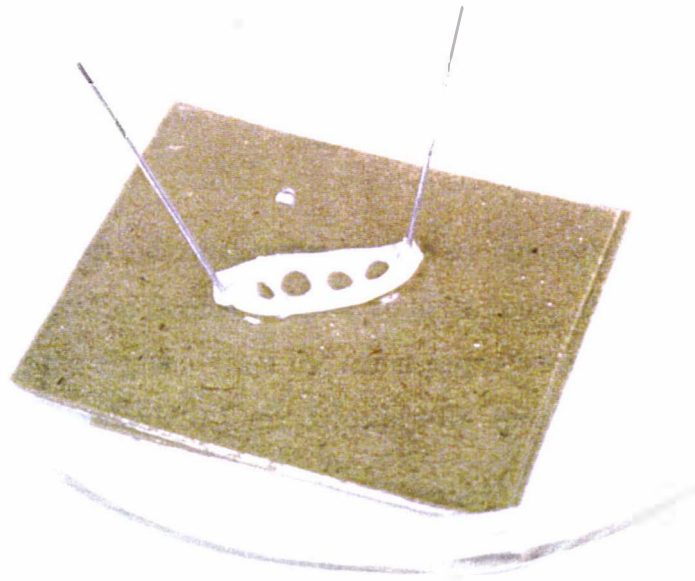
All animals were free from chronic metabolically debilitating disease, and had no history of corticosteroid, NSAID, or other recent drug therapy. Each middle carpal joint was inspected visually, and any joint with abnormal cartilage at or close to the sites of harvest was discarded.

##### 2. **Preparation of cartilage samples**

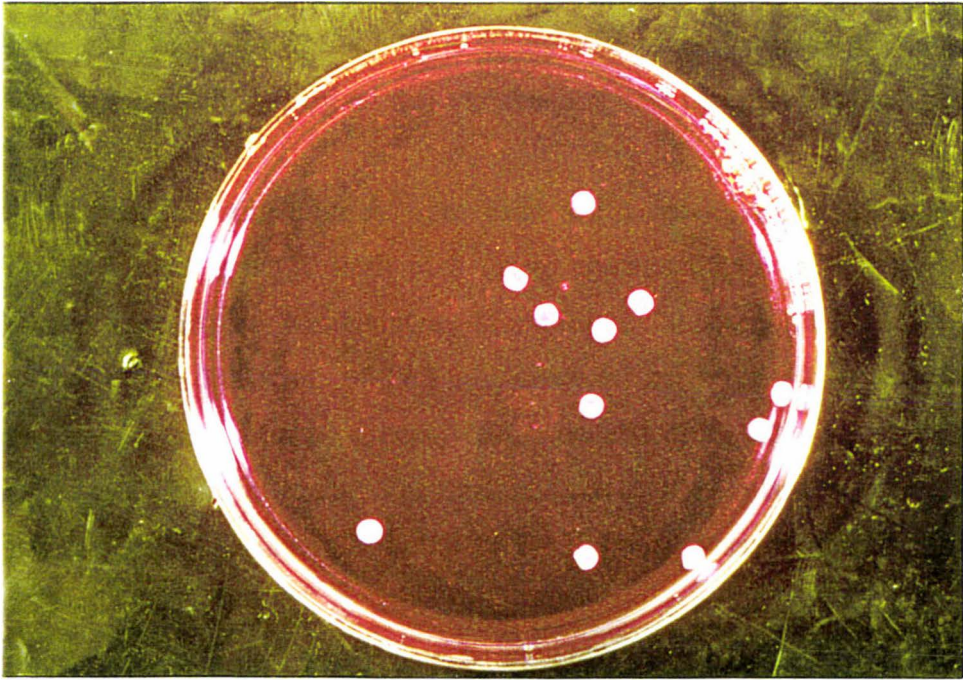
Within 6 hours of the death of each horse, both carpi were skinned and disarticulated at the middle carpal joint. The custom made cartilage chisel was used to harvest 4 mm wide full thickness strips of articular cartilage from the third and radial carpal bones (Figure 3.1). The cartilage from each site was placed in a separate screw-capped jar containing chilled (4°C) DMEM (pH 7.4) and PSK. A punch was used to cut as many 3 mm diameter explants as possible from the strips of cartilage from each site (Figure 3.2 and 3.3). The explants from the third carpal bones were processed first. Each was individually blotted dry and weighed on a Mettler balance (reproducibility 0.05 mg), then placed in 1.0 mL culture plate wells containing 0.5 mL DMEM (pH 7.4)



**Figure 3.1** Photograph of an open equine middle carpal joint showing the sites (top = third carpal bone, bottom = radial carpal bone) where the strips of articular cartilage were harvested from.



**Figure 3.2** Strip of articular artilage from which explants have been cut.



**Figure 3.3** Explants suspended in chilled DMEM awaiting further processing.

supplemented with 15% heat inactivated equine serum (ES). The explants rested directly on the well bottoms and no attempt was made to influence which surface faced uppermost. Explants were then incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and air.

Six explants from the radial carpal bones were randomly selected (non-cultured control group) and fixed in Bouins fluid for 16 to 24 hours, then transferred to 70% ethanol. The rest of the explants from the radial carpal bones were placed in 1.0 mL culture plate wells containing 0.5 mL DMEM+ES (pH 7.4) and incubated with the third carpal bone explants.

Both sets of explants were cultured for an initial 48 hour stabilising period with their media replaced after 24 hours.

### 3. Depo-Medrol (MPA) trial

Explants of articular cartilage from six horses were used in this study. A series of suspensions containing 0, 0.4, 4, and 40 mg mL<sup>-1</sup> of methylprednisolone acetate were prepared fresh each day by mixing variable amounts of Depo-Medrol<sup>25</sup> and PBS.

The 48 hour explant cultures from each site were randomly allocated into four control/treatment groups, until each group contained 25% of the total number of explants. Each explant was maintained for a further 72 hours in DMEM+ES (0.5 mL) to which either 0.025 mL of PBS (the controls) or 0.025 mL of one of the MPA suspensions (the treatment groups) had been added. Media and PBS/MPA were changed at 24 hour intervals. The medium used on the explants from the third carpal bones for the last 24 hours of culture (96-120 hours) was additionally supplemented with 5 μCi mL<sup>-1</sup> Na<sub>2</sub><sup>35</sup>SO<sub>4</sub>.

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<sup>25</sup> Depo-Medrol, 40 mg ml<sup>-1</sup> methylprednisolone acetate, Upjohn Inter-American Corporation.

#### 4. Phenylbutazone trial

Explants of articular cartilage from six different horses were used in this study. Phenylbutazone<sup>26</sup> (PBZ) was dissolved in 2M NaOH and added to DMEM during its preparation to form a DMEM + PBZ stock solution (pH 7.4). This stock solution was serially diluted with DMEM, and then had ES added, to give concentrations of 0, 2, 20, 200, and 2000  $\mu\text{g mL}^{-1}$  PBZ in DMEM + ES(15%).

Due to inadequate sample numbers, the explants allocated to the 2000  $\mu\text{g mL}^{-1}$  concentration of PBZ came from only five of the six horses for the histological study (radial carpal bones), and four out of the six horses for the amino sugar and <sup>35</sup>S incorporation studies (third carpal bones). In addition, four of the five 2000  $\mu\text{g mL}^{-1}$  treatment groups in the histological study contained one less explant than the other groups. These constraints withstanding, the 48 hour explant cultures from each site were randomly allocated into either four or five control/treatment groups, until each group contained 25% or 20% respectively of the total number of explants. Each group of explants was then maintained for a further 72 hours in culture medium (DMEM+ES) containing 0, 2, 20, 200, or 2000  $\mu\text{g mL}^{-1}$  PBZ, which was changed every 24 hours. The media used for the explants from the third carpal bones for the last 24 hours of culture (96-120 hours) were additionally supplemented with 5  $\mu\text{Ci mL}^{-1}$   $\text{Na}_2^{35}\text{SO}_4$ . Any explants from culture plate wells that showed any evidence of bacterial contamination were discarded.

#### B. POST-CULTURE PROCESSING

##### 1. Explants from the radial carpal bones

Immediately after culture termination the explants from the radial carpal bones were prepared for histological examination. Each group was fixed in Bouins fluid for 16 to 24 hours, then transferred to a solution of 70% ethanol in water and stored.

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<sup>26</sup> Phenylbutazone, gift from Ethical Agents Australia Ltd.

## 2. Explants from the third carpal bones

The explants from the third carpal bones were prepared for biochemical analysis. Each explant was subjected to the three wash procedure (as described in materials and methods: section one), and was then placed in a 5 mL pyrex screw-top vial containing 5 mL of 2N HCl, tightly sealed with a teflon lined screw-cap, and incubated at 100°C for 16 hours. The contents of any vials which had obviously leaked during the acid digest were discarded.

## C. ANALYTICAL TECHNIQUES

The amino sugar content of each explant was assayed as a measure of its glycosaminoglycan and proteoglycan content. The incorporation of <sup>35</sup>S during the final 24 hours of culture was used as a relative measure of glycosaminoglycan and proteoglycan synthesis. Histological assessment of cell death was used as measure of the relative cytotoxicity of the different doses/concentrations of the two drugs used.

### 1. Amino sugar assay

The amino sugar content of 0.6 mL amounts of the acid digested explants were assayed in duplicate according to the Gatt and Berman (1966) modification of the Elson and Morgan (1933) colorimetric method for the determination of amino sugars, as described in materials and methods: section one. The total amino sugar (AS) content of each explant was then calculated, divided by the initial wet weight of the explant, and expressed as  $\mu\text{g AS mg}^{-1}$ .

### 2. Scintillation counting procedure

From each of the acid digests 0.5 mL amounts were subjected to the scintillation counting procedure in duplicate, as described in materials and methods: section one. The calculated dpm occurring in each explant was then divided by the initial wet weight of the explant and expressed as  $\text{dpm mg}^{-1}$ .

### **3. Histological evaluation**

Each of the fixed explants was cut in half, and one half was processed and embedded in paraffin wax while the other half was stored. Sections ( $6\mu$ ) across the full diameter of each explant were cut and stained with alcian blue (pH 7.2), haematoxylin and eosin (H & E). A blinded assessment of both dead and total cell counts was made by an independent histologist, using the method described in materials and methods: section one. The percentage of cells dead in each explant was then estimated from these counts.

## **D. STATISTICAL METHODS**

### **1. Explants from the radial carpal bones**

For the explants from each horse, the percentage in each section classified as dead was calculated, then the mean dead cell percentage of the non-cultured group was subtracted with any negative results being rounded up to zero. The results from the explants of all six horses were then combined into their respective control/treatment groups, the distributions normalised by log transformation, and the means then analysed using Scheffe's pairwise comparisons ( $\alpha = 0.05$ ).

### **2. Explants from the third carpal bones**

The inherent variation in the amino sugar content and incorporation of  $^{35}\text{S}$  between the explants from different horses was controlled by dividing the results of each explant by the mean result of the control group from the same horse. The results from the explants of all six horses were then combined into their respective treatment groups. The distributions of the scintillation results were normalised by square root transformation. However, some heteroscedasticity still existed. Differences between treatment groups were analyzed by the method of linear contrasts (Sokal & Rohlf, 1981). For each drug and each parameter, three non-orthogonal combinations of treatment and control groups were used to examine the null hypotheses.

- (1) The drug has no effect.

$$U_{\text{control}} = U_{\text{treatments}}$$

- (2) The drug has no effect at the concentrations found in synovial fluid *in vivo*.

$$U_{\text{control}} = \frac{U_{20\mu\text{g mL}^{-1}} + U_{200\mu\text{g mL}^{-1}}}{2} \text{ (for MPA)}$$

$$U_{\text{control}} = \frac{U_{2\mu\text{g mL}^{-1}} + U_{20\mu\text{g mL}^{-1}}}{2} \text{ (for PBZ)}$$

- (3) The effect of the drug is not greater at higher concentrations than found *in vivo*.

$$\frac{U_{20\mu\text{g mL}^{-1}} + U_{200\mu\text{g mL}^{-1}}}{2} = U_{2000\mu\text{g mL}^{-1}} \text{ (for MPA)}$$

$$\frac{U_{2\mu\text{g mL}^{-1}} + U_{20\mu\text{g mL}^{-1}}}{2} = \frac{U_{200\mu\text{g mL}^{-1}} + U_{2000\mu\text{g mL}^{-1}}}{2} \text{ (for PBZ)}$$

All hypotheses were investigated at the  $\alpha = 0.05$  level of significance. The second and third null hypotheses were only investigated if the first was rejected.

## RESULTS

### A. DEPO-MEDROL (MPA) TRIAL

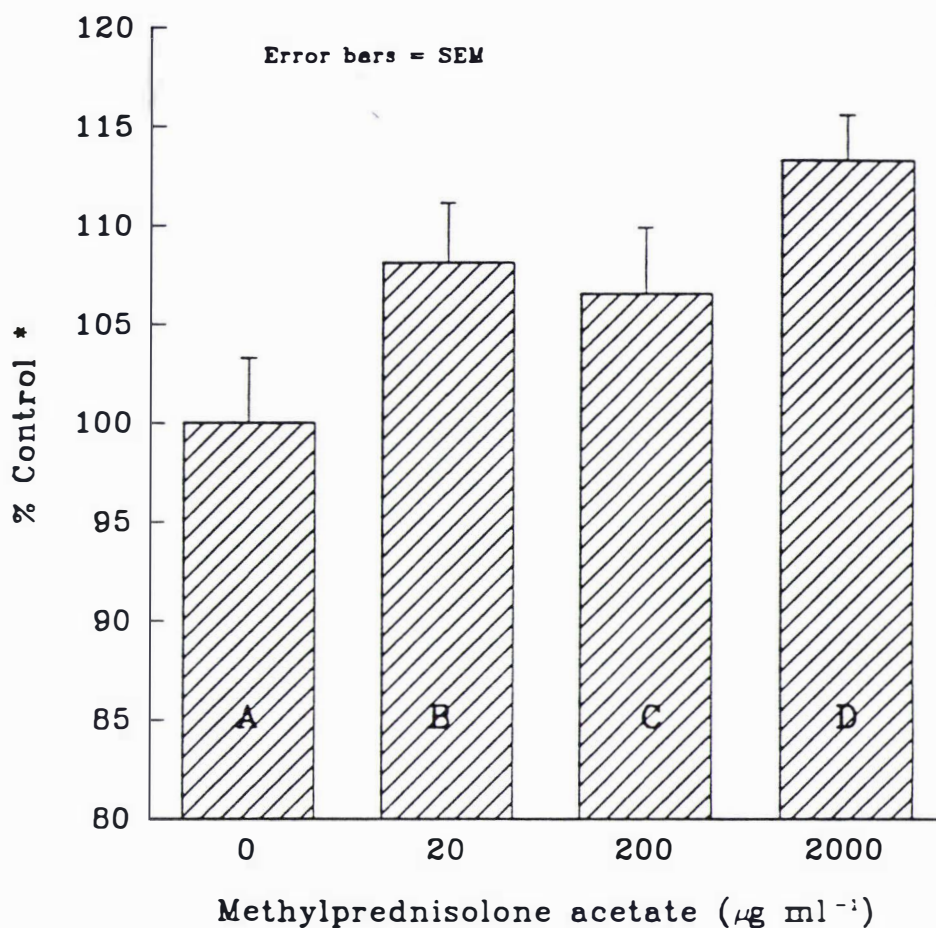
Seventeen of the 280 cultured explants from the third carpal bones were excluded from the trial due to evidence of bacterial contamination of their culture wells (4), or noticeable leakage from their sealed pyrex vials during acid digestion (13). Two of the 180 explants from the radial carpal bones were lost during processing.

#### 1. Effect of Depo-Medrol on amino sugar content

The dose related effect of Depo-Medrol on the amino sugar content of cultured TCB explants from six horses is summarised in Figure 4.1. When all the treatment groups were combined, the mean amino sugar content of the explants cultured in the presence of Depo-Medrol was higher than that of the cultured controls ( $F_{(1, 260)}$ ,  $p = 0.017$ ). The mean amino sugar content of the combined 20 and 200  $\mu\text{g mL}^{-1}$  treatment groups was also higher than that of the cultured controls, but the difference was not significant ( $F_{(1, 195)}$ ,  $p = 0.075$ ). The mean amino sugar content of the combined 20 and 200  $\mu\text{g mL}^{-1}$  treatment groups was also not significantly different from that of the 2000  $\mu\text{g mL}^{-1}$  treatment group ( $F_{(1, 200)}$ ,  $p = 0.1350$ ).

#### 2. Effect of Depo-Medrol on $^{35}\text{S}$ incorporation

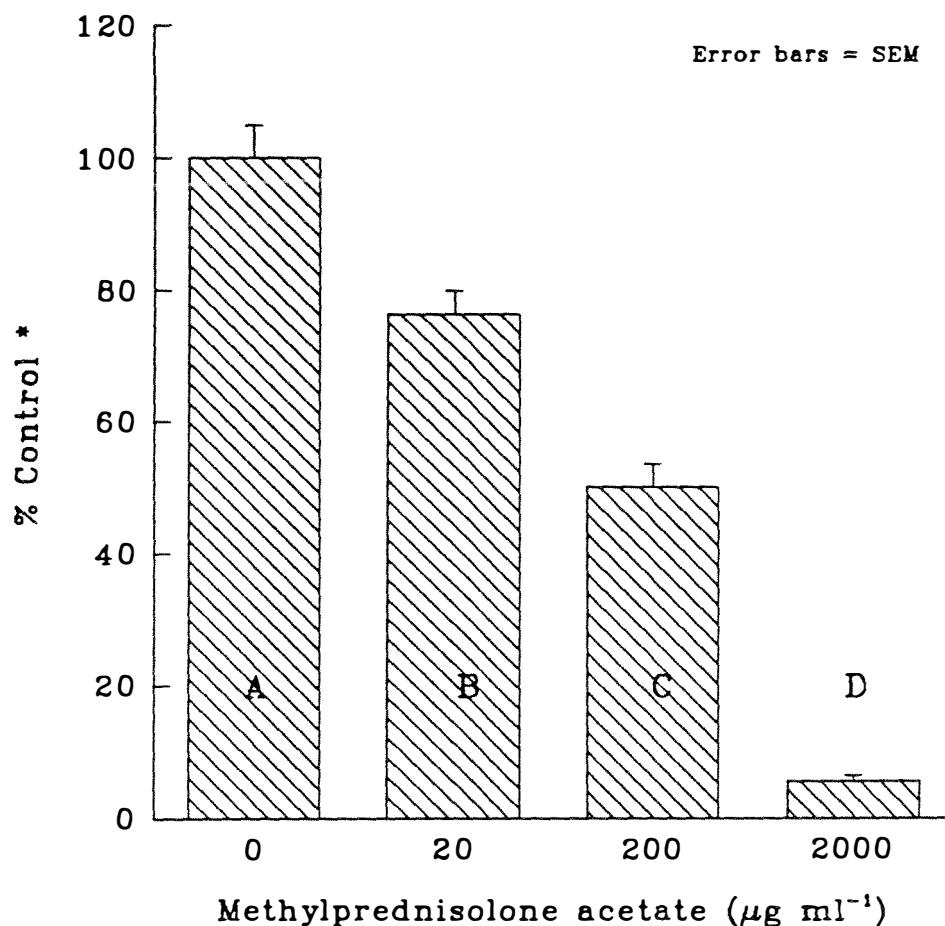
The dose related effect of Depo-Medrol on the incorporation of  $^{35}\text{S}$  by cultured TCB explants from six horses is summarised in Figure 4.2. Depo-Medrol depressed the incorporation of  $^{35}\text{S}$  in a dose dependant fashion. Incorporation of  $^{35}\text{S}$  by the cultured controls was higher than that by the combined treatment groups ( $F_{(1, 260)}$ ,  $p < 0.001$ ), and the 20 and 200  $\mu\text{g mL}^{-1}$  treatment groups ( $F_{(1, 195)}$ ,  $p < 0.001$ ). Furthermore the incorporation of  $^{35}\text{S}$  by the combined 20 and 200  $\mu\text{g mL}^{-1}$  treatment groups was also higher than that by the 2000  $\mu\text{g mL}^{-1}$  treatment group ( $F_{(1, 200)}$ ,  $p < 0.001$ ).



**Figure 4.1** Effect of Depo-Medrol on the amino sugar content of cultured explants of equine middle carpal joint articular cartilage.

\* The amino sugar  $\text{mg}^{-1}$  value of each explant was divided by the mean amino sugar  $\text{mg}^{-1}$  value of the control group from the same horse. The combined results from all the horses were then separated from the four control/treatment groups, averaged and expressed as percentages  $\pm$  SEM (number of explants in each group: A = 61, B = 67, C = 69, and D = 66).

Groups B, C, and D combined are significantly different from group A ( $p = 0.017$ ). However, group B and C combined are not significantly different from either group A ( $p = 0.075$ ) or group D ( $p = 0.135$ ).



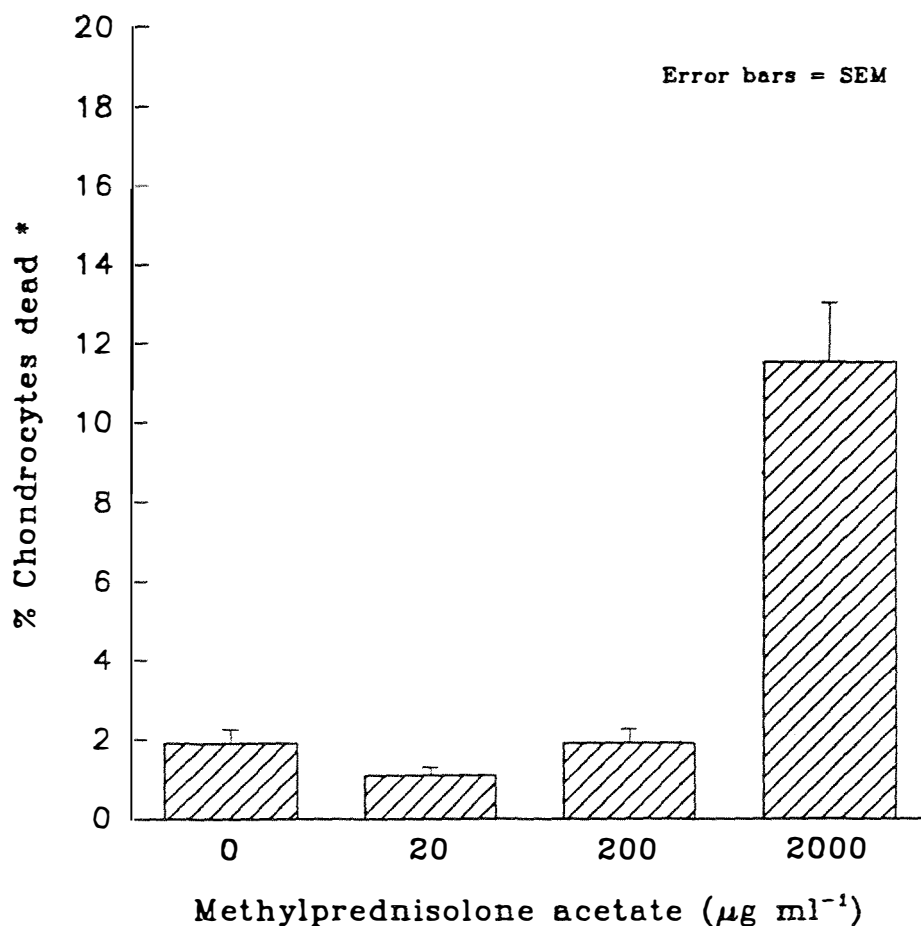
**Figure 4.2** Effect of Depo-Medrol on  $^{35}\text{S}$  incorporation by cultured explants of equine middle carpal joint articular cartilage.

\* The  $\text{dpm mg}^{-1}$  value of each explant was divided by the mean  $\text{dpm mg}^{-1}$  value from the same horse. The combined results from the explants of all the horses were then separated out into the four control/treatment groups, averaged, and expressed as percentages  $\pm$  SEM (number of explants in each group: A = 61, B = 67, C = 69, and D = 66).

Groups B, C, and D combined are significantly different from group A ( $p < 0.001$ ). Groups B and C combined are also significantly different from group A ( $p < 0.001$ ), and are significantly different from group D ( $p < 0.001$ ) as well.

### 3. Effect of Depo-Medrol on chondrocyte viability

The dose related effect of Depo-Medrol on the viability of chondrocytes within cultured RCB explants from six horses is summarised in and Figure 4.3. Depo-Medrol affected the percentage of pyknotic nuclei found only at the 2000  $\mu\text{g mL}^{-1}$  concentration ( $p < 0.001$ ).



**Figure 4.3** Effect of Depo-Medrol on the viability of chondrocytes within cultured explants of equine middle carpal joint articular cartilage.

\* The percentage of chondrocytes determined as dead in each cultured explant was reduced by the average percentage of chondrocytes found dead in the non-cultured group of explants from the same horse. Any resulting negative percentages were rounded up to zero, then all results were combined into the four control/treatment groups and averaged (number of explants in each group: 0 = 35, 20 = 35, 200 = 36, and 2000 = 36).

The percentage of chondrocytes dead in the 2000 µg ml<sup>-1</sup> group was significantly different from the other groups ( $p < 0.001$ ).

## B. PHENYLBUTAZONE (PBZ) TRIAL

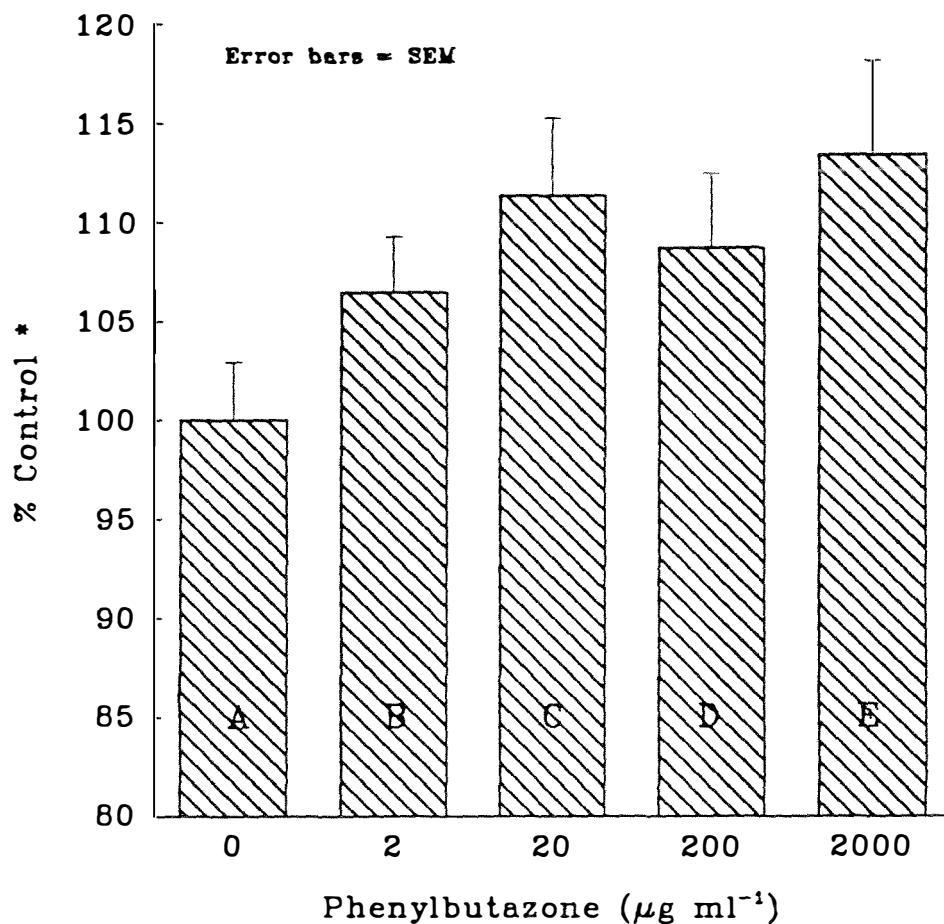
Fifteen of the TCB explants, out of the 296 cultured, were excluded from the trial due to evidence of bacterial contamination of the culture media (3), or noticeable leakage from the sealed pyrex vials during acid digestion (12). Because insufficient explants could be harvested from the radial carpal bones slightly lower numbers of explants were allocated to the non-cultured group and 2000  $\mu\text{g mL}^{-1}$  treatment group. One of the 185 explants cultured was lost during processing.

### 1. Effect of PBZ on amino sugar content

The effect of different concentrations of PBZ on the amino sugar content of cultured TCB explants from six horses is summarised in Figure 4.4. When all the treatment groups were combined, the mean amino sugar content of the explants cultured in the presence of PBZ was higher than that of the cultured controls ( $F_{(1, 277)}$ ,  $p = 0.011$ ). The mean amino sugar content of the combined 2 and 20  $\mu\text{g mL}^{-1}$  treatment groups was also higher than that of the cultured controls ( $F_{(1, 183)}$ ,  $p < 0.01$ ). However, there was no significant difference between the amino sugar content of the combined 2 and 20  $\mu\text{g mL}^{-1}$  treatment groups and the combined 200 and 2000  $\mu\text{g mL}^{-1}$  treatment groups ( $F_{(1, 216)}$ ,  $p = 0.59$ ).

### 2. Effect of PBZ on $^{35}\text{S}$ incorporation

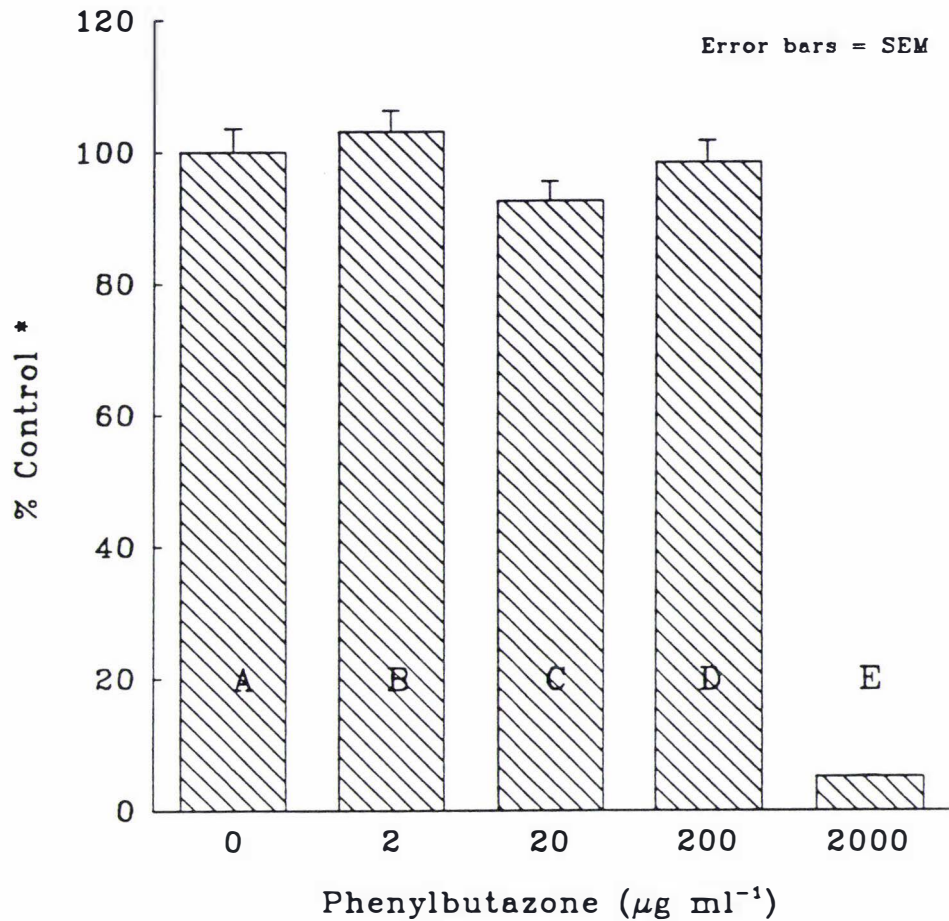
The effect of different concentrations of PBZ on the incorporation of  $^{35}\text{S}$  by cultured TCB explants from six horses is summarised in Figure 4.5. Only the 2000  $\mu\text{g mL}^{-1}$  PBZ treatment group had a significantly lower mean incorporation of  $^{35}\text{S}$  than the control group ( $F_{(1, 216)}$ ,  $p < 0.001$ ).



**Figure 4.4** Effect of phenylbutazone on the amino sugar content of cultured explants of equine middle carpal joint articular cartilage.

\* The amino sugar  $\text{mg}^{-1}$  value of each explant was divided by the mean amino sugar  $\text{mg}^{-1}$  value of the control group from the same horse. The combined results from all the horses were then separated into four control/treatment groups, averaged and expressed as percentages  $\pm$  SEM (number of explants in each group: A = 62, B = 61, C = 62, D = 60, E = 36).

Groups B, C, D, and E combined are significantly different from group A ( $p = 0.011$ ). Groups B and C combined are also significantly different from group A ( $p < 0.001$ ), but are not significantly different from groups D and E combined ( $p = 0.59$ ).



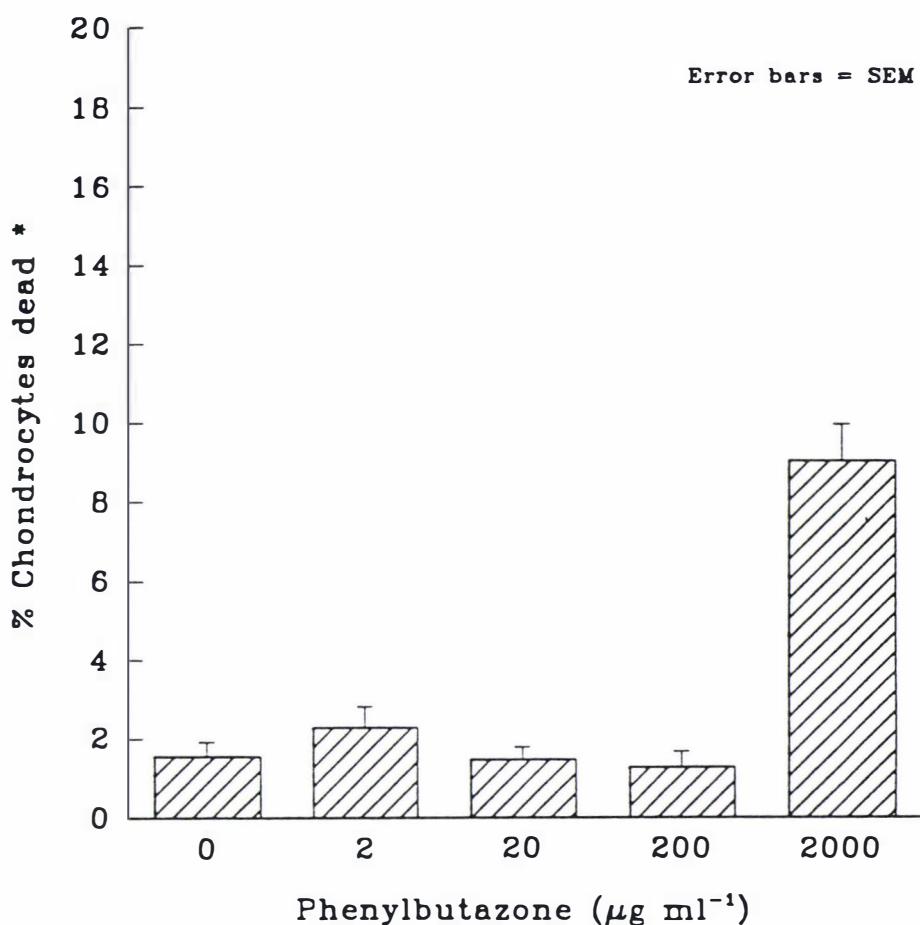
**Figure 4.5** Effect of phenylbutazone on  $^{35}\text{S}$  incorporation by cultured explants of equine middle carpal joint articular cartilage.

\* The  $\text{dpm mg}^{-1}$  value of each explant was divided by the mean  $\text{dpm mg}^{-1}$  value of the control group from the same horse. The combined results from the explants from all the horses were then separated into five control/treatment groups, averaged and expressed as percentages  $\pm$  SEM (number of explants in each group: A = 62, B = 61, C = 62, D = 60, and D = 36).

Only the  $2000 \mu\text{g ml}^{-1}$  phenylbutazone treatment group had a significantly lower mean  $^{35}\text{S}$  incorporation than the other groups ( $p < 0.001$ ).

### 3. Effect of PBZ on chondrocyte viability

The effect of different concentrations of PBZ on the viability of chondrocytes within cultured RCB explants from six horses is summarised in Figure 4.6. PBZ affected the percentage of pyknotic nuclei found only at the 2000  $\mu\text{g mL}^{-1}$  concentration ( $p < 0.01$ ).



**Figure 4.6** Effect of phenylbutazone on the viability of chondrocytes within cultured explants of equine middle carpal joint articular cartilage.

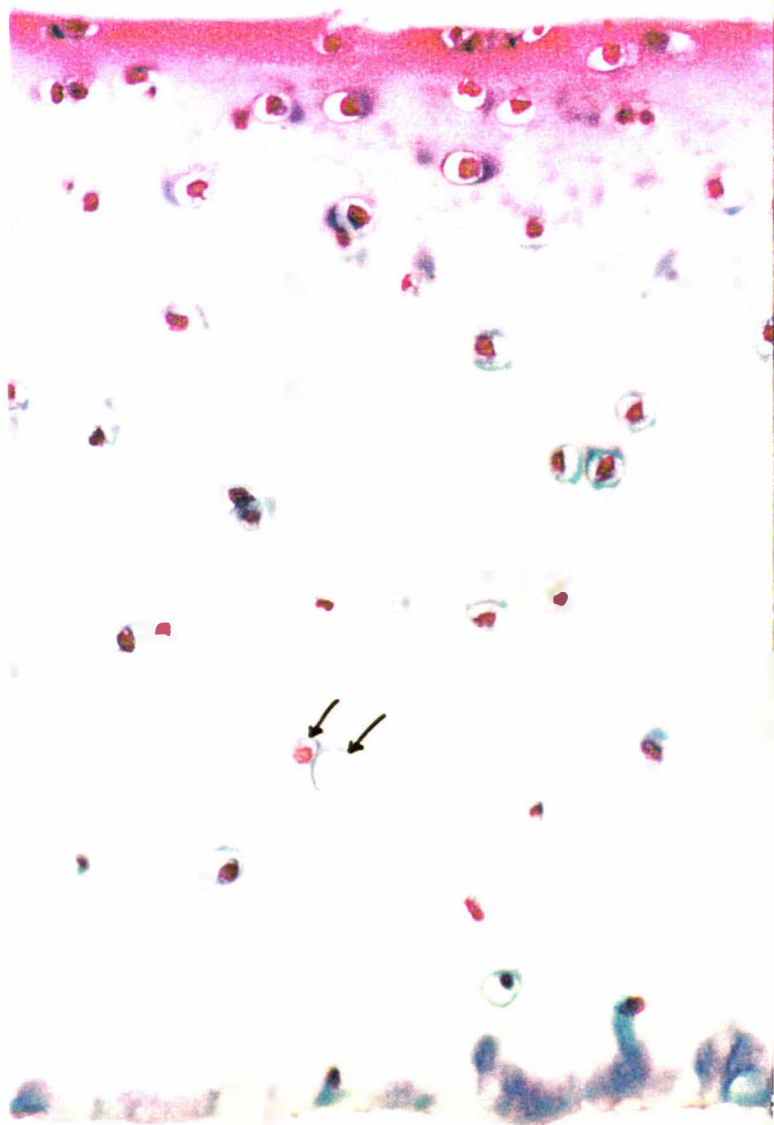
\* The percentage of chondrocytes determined as dead in each cultured explant was reduced by the average percentage of chondrocytes found dead in the non-cultured group of explants from the same horse. Any resulting negative percentages were rounded up to zero, then all results were combined into the five control/treatment groups and averaged (number of explants in each group: 0 = 29, 2 = 32, 20 = 32, 200 = 32, and 2000 = 28).

Only the 2000  $\mu\text{g ml}^{-1}$  phenylbutazone treatment group had a significantly greater mean percentage of chondrocytes that died during culture than the other groups ( $p < 0.01$ ).

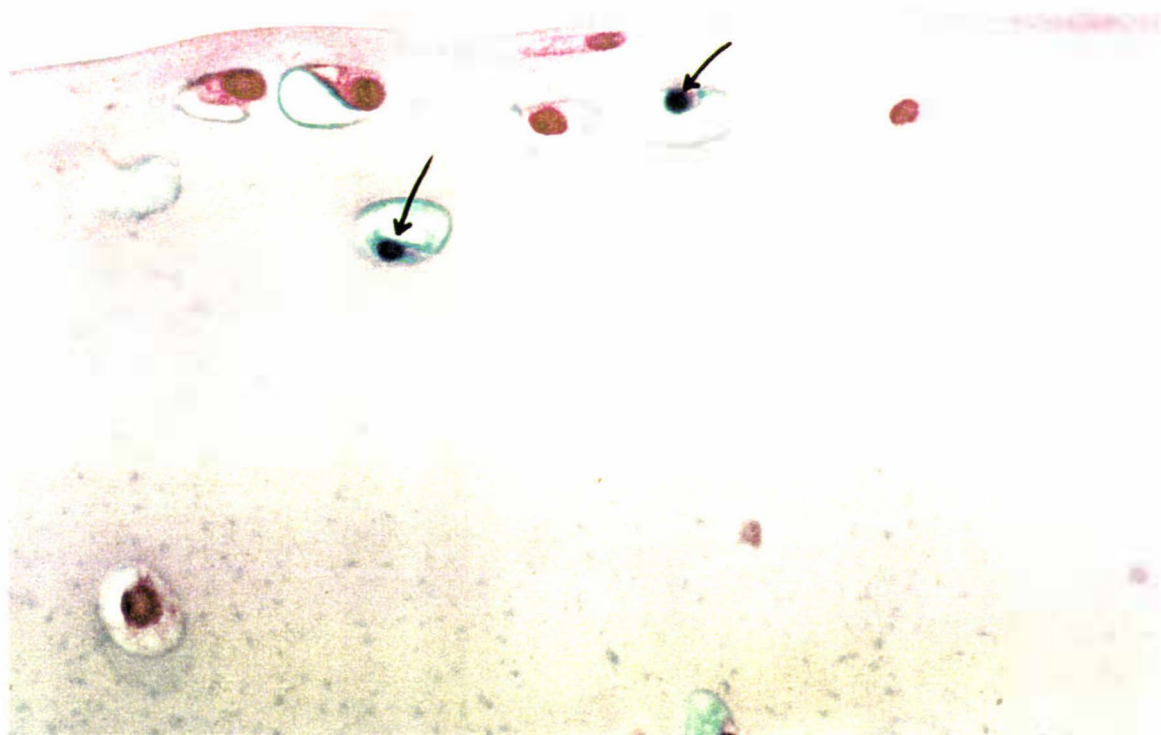
### C. HISTOLOGICAL TRIAL OBSERVATIONS

The staining characteristics, chondrocyte morphology, and size of the sections examined varied widely both within groups of explants from the same horse and between similar groups of explants from different horses. The appearance of individual cells in any one section varied substantially with nuclei of many shapes and sizes seen. Empty lacunae were seen in all sections. In some cases, the missing nuclei appeared to have been displaced into the surrounding matrix during processing (Figure 4.7). The intensity of the haematoxylin staining of the nuclei also varied between similar sections. However, the nuclei tended to be darker and smaller in the sections exposed to the highest drug concentrations (Figure 4.7). Cytoplasmic vacuolation was more apparent subjectively in the sections with higher dead cell percentages. Cell death was most commonly seen close to the articular surface and at the ends of the sections (Figure 4.8). Only a small number of closely associated clusters of chondrocytes were seen in any one section, and no obvious difference in their number was noticed between any of the drug concentrations and the controls. The clusters seen within the transitional and radiate zones appeared to be contained within separately delineated ovoid structures (Figure 4.9).

The sections cut from the non-cultured group of explants consistently had the most intense alcian blue staining. However, even in this group the staining intensity was not always consistent between sections from the same horse (Figure 4.10), and there was substantial variation between sections from different horses. Sections of the cultured control group of explants tended to stain least with alcian blue, but no difference could be observed between the alcian blue staining intensities of explants exposed to the different concentrations of either Depo-Medrol or PBZ. In both drug trials interterritorial staining intensities appeared to be affected before territorial staining intensities.



**Figure 4.7** Photomicrograph of an empty lacunae (↗) with the displaced nucleus sitting adjacent (1000x magnification).



**Figure 4.8** Photomicrograph of two dead chondrocytes (↗) close to the articular surface (4000x magnification).

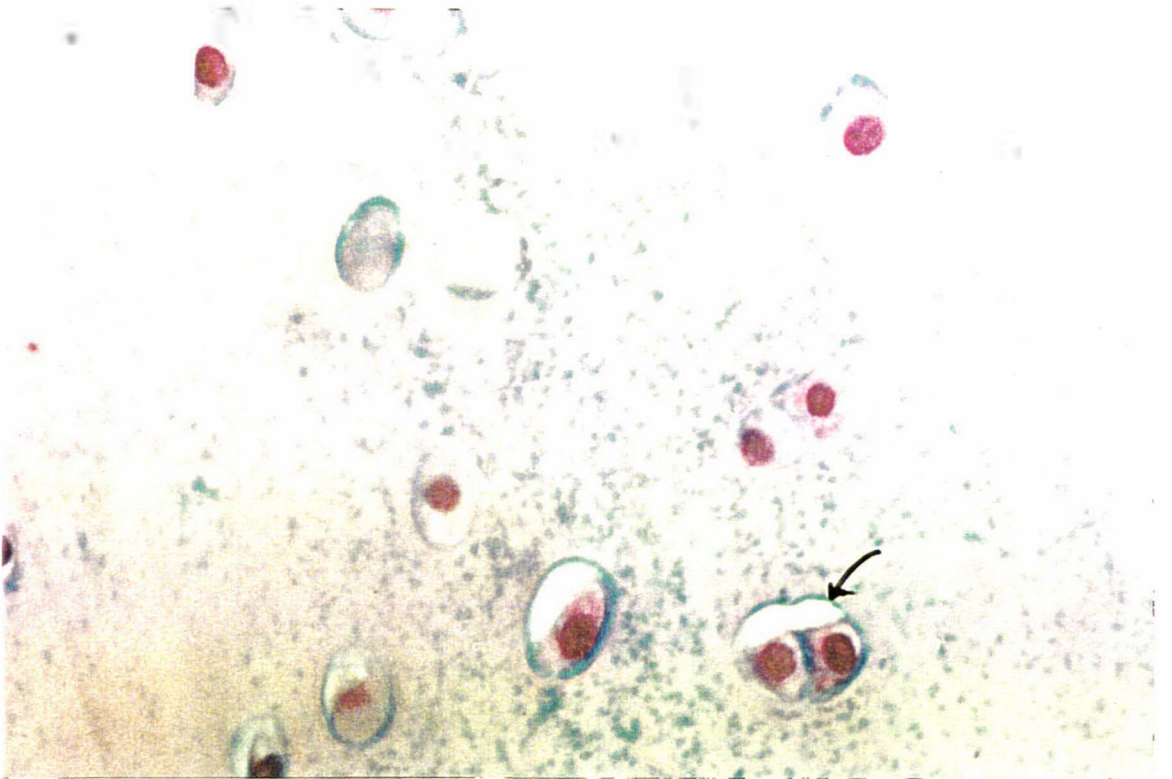
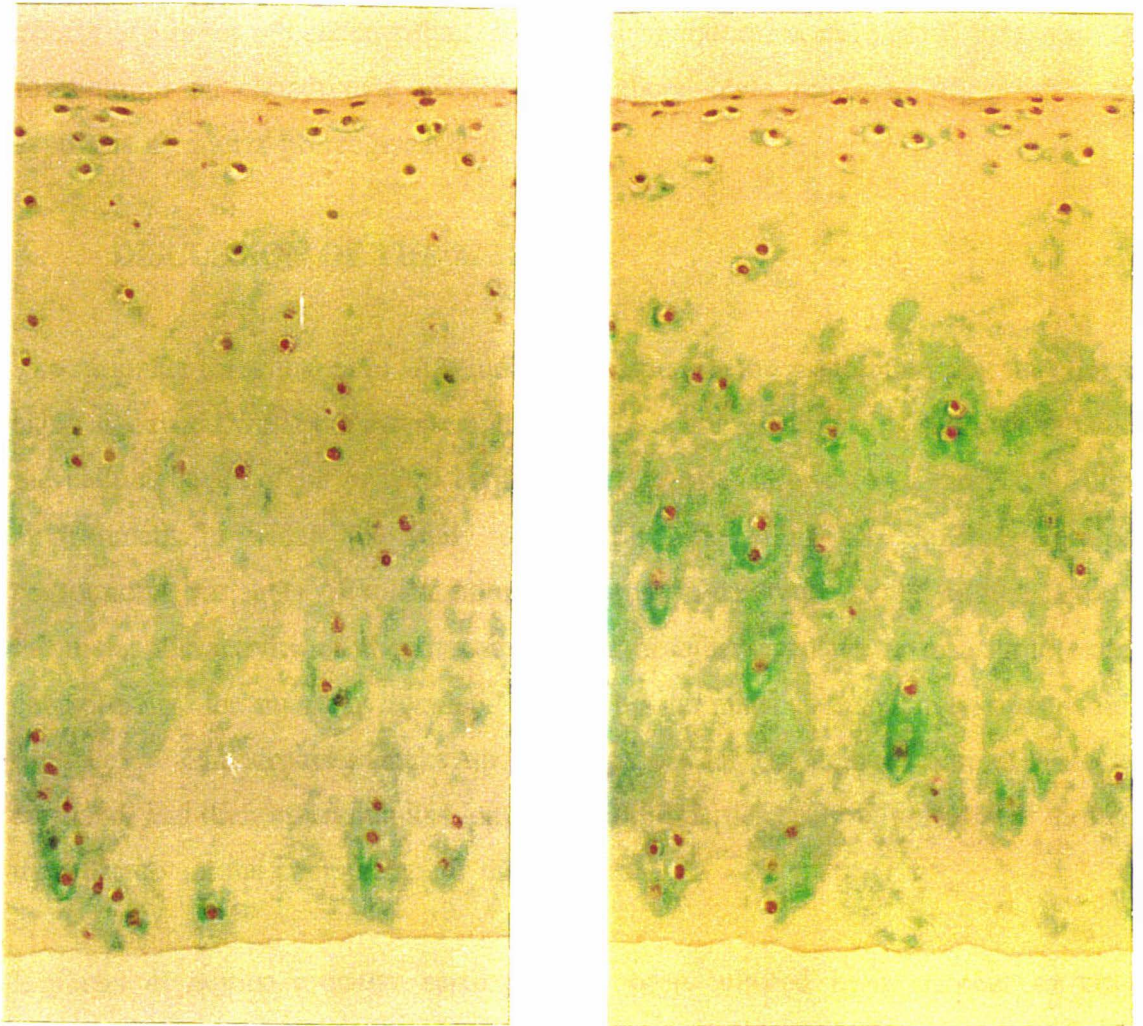


Figure 4.9 Photomicrograph of a chondrocyte cluster (↗) (4000x magnification).



**Figure 4.10** Comparison of the variation in proteoglycan content, as shown by alcian blue staining, of two sections cut from non-cultured explants harvested from the radial carpal bone of the same horse (400x magnification).

## DISCUSSION

The objectives of this investigation were to (a) establish and validate a tissue culture model of equine carpal articular cartilage, and (b) use this model to examine the direct effects of a series of concentrations/doses of phenylbutazone and a methylprednisolone formulation on chondrocyte viability, and chondrocyte mediated degradation and synthesis of matrix proteoglycans.

### A. DISCUSSION OF THE METHODS

The rationale for using an *in vitro* model was based on the assumption that the clinical signs associated with both disease and drug effects are reflections of actions exerted at the cellular level. The *in vitro* maintenance of tissue allows for a more controlled environment in which the study of specific interactions can be isolated from confounding variables. Explant culture was used in deference to cell culture because the metabolic activity of chondrocytes within articular cartilage is to a large extent controlled by the microenvironment created by the matrix immediately surrounding each cell. Chondrocytes make up less than 5% of the composition of articular cartilage, and their effects on its biomechanical properties are solely mediated through their effects on the components of the matrix.

Explants of equine articular cartilage have been cultured by a number of other researchers for durations of up to 13 days (Pool *et al.*, 1980; Chunekamrai *et al.*, 1989; Caron *et al.*, 1991; MacDonald *et al.*, 1992; Caron *et al.*, 1993). The use of explants maintained many of the normal tissue interactions present in joints, but also resulted in structurally abnormal surfaces being exposed, and possibly changed normal nutrient and metabolic gradients. In addition, the large inherent variation between explants in cellular and proteoglycan content meant large sample numbers were required to ensure clinically significant treatment differences could be shown. The site of harvest, and the size and shape of the explants, were chosen to maximise the number of explants while minimising between-explant variation and the cut surface area to volume ratio of each explant.

The explants came from horses destined for destruction, so strictly speaking they could not be considered to be representative of the articular cartilage of thoroughbreds in training. Nevertheless, all joints used were considered healthy and were without any obvious degenerative changes. Sample allocation was randomised with the exception that fixed sample sizes were used and the highest concentration group had its number of explants reduced if any shortage arose. Large sample sizes minimised any bias this may have had on the results. The conditions from time of death until culture initiation adversely affect chondrocyte metabolism. Explants were thus allowed to recover and acclimatise to the *in vitro* culture conditions for 48 hours before the drugs to be tested were added. In this way the effects of the drugs were thought to be more representative of their effects on pathological processes as they might occur *in vivo*, rather than of their modifying effects on the stresses associated with culture initiation. Radiolabelling was initiated only after 96 hours of culture to allow both a stabilisation of synthetic rates and time for the drugs to have exerted their effects.

Serum was considered an essential part of the culture media as it provided a source of growth factors, cholinesterases, and albumin. Adult equine serum was used to ensure the types and concentrations of growth factors present closely resembled those of the animals from which the explants originated. Growth factors were necessary to maintain proteoglycan synthesis for the duration of the culture, while cholinesterases were necessary to convert methylprednisolone acetate (MPA) into the active methylprednisolone (MP) form. However, it was not known what percentage of the cholinesterases survived the heat inactivation of the serum. As PBZ is normally extensively protein bound (>98%), albumin was required to ensure the chondrocytes were exposed only to the fraction of PBZ that would 'normally' exist in the unbound form.

The use of serum in the media meant the amino sugar content of the released proteoglycans could not be assayed by the method of Gatt & Berman (1966) (see Materials & Methods: Section One). As a result there was no direct measure of proteoglycan catabolism. However, the final amino sugar content of each explant reflected both the anabolic and catabolic processes occurring in the explant during the

culture period. Thus, conclusions about catabolism could still be inferred from comparisons of the final amino sugar content and the relative rate of proteoglycan synthesis of each treatment group to those of the control group.

An amino sugar assay was chosen to measure relative changes in the proteoglycan content of cultured explants of equine articular cartilage primarily because both of the major types of glycosaminoglycan present have equal proportions. Contribution of amino sugars from the hyaluronic acid and glycoprotein fractions of articular cartilage was thought likely to be insignificant in comparison to the amounts released from the proteoglycans. The use of an amino sugar assay also allowed for the use of a total digestion of each explant. Total digestion of the explants was considered preferable to the use of proteoglycan extraction techniques which leave a variable proportion of the proteoglycan content of each explant behind.

The use of uronic acid assays, and to some extent the use of cationic dyes, neglects the contribution of the keratansulphate component of equine articular cartilage. The ratio of chondroitin sulphate to keratansulphate is not consistent between horses, joints, sites within a joint, or even with distance from the articular surface. Furthermore, keratansulphate side-chains tend to be concentrated on the hyaluronic acid binding end of proteoglycan molecules while chondroitin sulphate predominates on the distal two thirds. Proteolytic cleavage part of the way along a proteoglycan molecule could possibly reduce the proportion of chondroitin sulphate relative to the proportion of keratansulphate remaining restrained within cultured explants of articular cartilage. Cationic dyes can also be affected by the type and length of the glycosaminoglycans remaining bound to the hyaluronic acid molecule.

The determination of individual amino sugar concentrations by ion chromatographic methods may have been more precise than a non-specific amino sugar assay (Vachon *et al.*, 1990), but the availability and expense of this technology precluded its use for these experiments. Although time consuming, use of the Gatt & Berman (1966) amino sugar assay required minimal sample manipulation and was relatively simple to perform. The absorbances produced by galactosamine and glucosamine at 524 nm

were not identical, but the differences were substantially smaller than those seen with the use of dimethylmethylene blue assays. Furthermore, interference in the assay by other components of equine articular cartilage was shown not to be a major concern (see Materials & Methods: Section One).

Incorporation of  $^{35}\text{S}$  has commonly been used for comparing rates of proteoglycan synthesis between explants of articular cartilage. On average, both chondroitin sulphate and keratansulphate contain one sulphate group per disaccharide unit. The addition of the sulphate group occurs relatively late in the synthesis of these glycosaminoglycans and accounts for most of the sulphate fixation occurring in articular cartilage. The sulphate groups attached to chondroitin sulphate and keratan sulphate, with their associated negative charges, are responsible for much of the physicochemical and resulting biomechanical properties of articular cartilage.

The use of cationic dyes such as safranin-O and alcian blue for quantitative histological comparisons of proteoglycan content, and or distribution changes, is subject to poor repeatability (Hunziker *et al.*, 1992). Staining intensities can be affected by variable proteoglycan losses occurring during routine fixation and processing, as well as by the types and lengths of the glycosaminoglycan side-chains remaining bound to the protein cores of the proteoglycan molecules. Ideally, large numbers of explants would need to be fixed in the presence of a cationic dye such as ruthenium then processed, cut, and stained as a single batch to allow even semi-quantitative comparisons to be made (Hunziker *et al.*, 1992). For these reasons very limited conclusions were drawn about changes in proteoglycan content from the histological study.

The use of wet weights to standardise the cellular and proteoglycan content of explants precluded use of part of the same explant for histological sectioning. Explants were thus used from a different site in the same joints, and interpretations of the combined results assumed explants from both sites behaved in a similar manner. The histological results confirmed much of the variability shown to exist between the explants by the biochemical analyses. Cell numbers varied greatly between sections as did the appearance of the individual chondrocytes in the various zones. Some of this variation

was due to differences in sampling depths, but most was caused by inherent variation in cellular distributions and characteristics known to occur within even small areas of a joint. It was concluded that a great deal of care must be exercised in interpreting differences between histological sections, even from the same site let alone from different joints and horses. Expressing dead cell numbers as percentages of the total number of nucleated cells removed some of the variation caused by the greatly differing cell numbers in the explants. The percentage of chondrocytes found dead in each explant was then reduced by the average percentage of chondrocytes found dead in the non-cultured group of explants from the same horse. This was an attempt to exclude that proportion of chondrocytes whose death may have been associated with variations in processing times, and was probably not due to either culture or drug effects. When the individual results were combined into their respective control/treatment groups the distribution of results within most of the groups tended to be skewed towards zero. However, a log transformation normalised the distributions and standardised the variances of the different groups.

Histological assessment of nuclear morphology was considered the most objective and definitive measure of the cytotoxic affects of different drug amounts/concentrations. In the preliminary studies substantial variation was seen in both the cytoplasmic and nuclear staining characteristics of sections cut from similarly treated explants. Classification of cell viability purely on the nuclear morphology at 10,000 x magnification produced the most definitive results. Exclusion of anuclear cells from both the total and dead cell counts may have underestimated dead cell percentages, but was used because there was no way of determining what percentage of the 'empty lacunae' were due to sectioning orientation or artefact. Degenerative nuclear changes have been used by other researchers as an assessment of cytotoxicity (Shoemaker *et al.*, 1992; Gustafson *et al.*, 1992).

Explant culture provided a closed system in which interactions between drug and tissue could be studied under controlled conditions which involved minimal changes to either cellular phenotypic expression or drug-matrix interactions. Explants remained metabolically active and viable, but there was a net loss of proteoglycans during

culture. One of the initial aims of using an *in vitro* model was to assess what effects the drugs to be tested had on 'normal' articular cartilage. However, it was obvious from preliminary experimental work that the assumption of normality could not be applied further than the source of the tissue. The metabolic behaviour of the explants more closely resembled the *in vivo* response of articular cartilage to injury. During culture there were reductions in both cell viability and proteoglycan content, while the rate of proteoglycan synthesis increased markedly. This was so especially for a period immediately following culture initiation, but thereafter rates of proteoglycan synthesis and catabolism were more constant, although still resulting in a net depletion of proteoglycans from the explants.

## B. DISCUSSION OF THE RESULTS

Methylprednisolone acetate (MPA) and phenylbutazone (PBZ) are the most common steroid and non-steroidal anti-inflammatory drugs used for treatment of joint injury in equine athletes. Their soft tissue mediated clinical effects are well recognised. Whether or not they also confer some degree of chondroprotection is actively debated (Tobin *et al.*, 1986; McIlwraith & Vachon, 1988). Furthermore, there is some evidence to suggest their use may actually potentiate the progression of joint deterioration (Gabel, 1978; Owen, 1980; Chunekamrai *et al.*, 1989; Trotter *et al.*, 1991). Both the types and mechanisms of their effects on articular cartilage are subjects of some conjecture and much controversy. Despite the controversy, relatively few controlled *in vivo* trials have investigated the effects of MPA or PBZ on equine articular cartilage. Interpretation of these trials has been hindered by small sample numbers, a wide range of confounding variables, and the limited number and type of investigative procedures performed.

Several investigations have attempted to reduce the variation in composition between explants by analysing large groups together. As a consequence most of the experiments have been restricted to very small sample sizes. In the present experiment inherent within-horse and between-horse variation in proteoglycan and cellular content was reduced by dividing the results of each explant by its initial wet weight, then further dividing these results by the mean of the control group from the same horse. In this way sample numbers were maximised while some of the inherent variation was controlled. The statistical power of each comparison was further increased by analysing the results using the method of linear contrasts, which allowed examination of the three major hypotheses by nonorthogonal combinations of the treatment and control groups. However, lack of statistical power caused by the large inherent variation between explants was still a major limitation to the extent to which the amino sugar content results could be analyzed and interpreted.

For each explant, amino sugar content was used as a measure of proteoglycan content (Glade *et al.*, 1983; Richardson & Clark, 1990; Vachon *et al.*, 1991), <sup>35</sup>S incorporation

as a measure of the rate of proteoglycan synthesis (Maroudas & Evans, 1974; Tyler, 1985; Chunekamrai *et al.*, 1989), and the number of pyknotic nuclei as a measure of cell death (Ham, 1974; McIlwraith & Van Sickle, 1981; Shoemaker *et al.*, 1992). The two lowest drug concentrations were chosen to encompass the range likely to be present in equine synovial fluid following the administration of clinically recommended doses (Lehmann *et al.*, 1981; Autefage *et al.*, 1986; Lees *et al.*, 1987). However, the extent to which the methylprednisolone acetate (MPA) doses dissolved in the culture media, or were hydrolysed to the pharmacologically active methylprednisolone (MP) form, were unknown.

A commercial formulation of MPA was used, as opposed to MPA or MP alone, to ensure the results more closely approximated the clinical scenario. The extents to which the MPA doses dissolved in the culture media, or were hydrolysed to the pharmacologically active MP form by the serum colinesterases, were unknown. No attempt was made to control for any effects the excipients of Depo-Medrol may have had on the experiments. Accordingly, the conclusions drawn are restricted to statements about the effect of Depo-Medrol on cultured explants of equine carpal articular cartilage, although the possible mechanisms of action are discussed.

The  $^{35}\text{S}$  scintillation results were more variable than the amino sugar content results but differences between treatment groups were also expected to be larger. Statistical power was thus not a limiting factor in the analysis of these results, but for the sake of interpretation both sets of results were analyzed in a similar fashion. To facilitate this a number of different transformations were tried in an attempt to normalise the sample population distributions of the different groups, and to standardise their variances. None of these transformations resulted in distributions which satisfied Bartlett's test for equal variances (see Appendix), but a square root transformation resulted in the lowest  $X^2$  value for Bartlett's test. The variances associated with the  $2000\ \mu\text{g mL}^{-1}$  treatment groups of both drugs were the only ones markedly different from the others. For parametric analyses, the assumption of equal variances is most critical when sample sizes are small or markedly different (Sokal & Rohlf, 1981). The size of the samples and the differences between the groups were both large enough that we did not consider

the violation of equal variances would significantly affect the results at the level they were to be interpreted.

The presence of Depo-Medrol in the culture media caused a dose related suppression of proteoglycan synthesis in the explants. The permanence of this suppression was not assessed. The suppressive effect of Depo-Medrol was significant at clinically applicable doses of MPA (20-200  $\mu\text{g mL}^{-1}$ ), and was significantly greater at the 2000  $\mu\text{g mL}^{-1}$  dose. Paradoxically, the presence of Depo-Medrol in the culture media reduced the depletion of proteoglycans from the explants. There was limited evidence ( $p = 0.075$ ) to suggest this effect was present at the clinically applicable doses, supported by the fact that there was less evidence to suggest the effect was any greater at the 2000  $\mu\text{g mL}^{-1}$  dose ( $p = 0.135$ ). Theoretically, proteoglycan depletion can arise either from increase in catabolism or decrease in synthesis. Because rate of proteoglycan synthesis appeared to have little influence on the final proteoglycan content of the explants, it was concluded that any reductions in proteoglycan content were mainly the result of catabolic processes. Furthermore, the positive correlation between cell death and proteoglycan content suggested increases in proteoglycan catabolism were predominantly due to metabolically active processes. Depo-Medrol was cytotoxic at only the 2000  $\mu\text{g mL}^{-1}$  dose rate. Lysosomal enzyme release associated with cell death would not seem to have been a major cause of proteoglycan depletion.

The effect of Depo-Medrol on the synthesis of proteoglycans in equine articular cartilage, as measured by radiolabel incorporation, has previously been reported by only two studies (Chunekamrai *et al.*, 1989; Fubini *et al.*, 1993). Fubini *et al.* (1993) reported that there was no significant difference between the rate of incorporation of  $^{35}\text{S}$  by articular cartilage from control joints versus that from joints treated with Depo-Medrol 21 days previous. In contrast, Chunekamrai *et al.* (1989) found that following 8 weekly intra-articular administrations of Depo-Medrol, proteoglycan synthesis was reversibly suppressed for periods in excess of 8 weeks. Similar findings have also been reported in other species subjected to multiple and frequent administrations of large doses of sparingly soluble steroid preparations (Mankin & Conger, 1966; Mankin

*et al.*, 1972; Behrens *et al.*, 1975). Generally, the degree and duration of suppression has been proportional to the number, size, and frequency of doses.

The reduced proteoglycan depletion in this study is in agreement with the *in vitro* findings of Jacoby (1976) and Tyler *et al.* (1982), but not with those of Pool *et al.* (1981). However, the later were part of a preliminary report which gave few details of the methods used. *In vivo* studies of MPA on the proteoglycan content of equine articular cartilage (Chunekamrai *et al.*, 1989; Trotter *et al.*, 1991; Shoemaker *et al.*, 1992) have all reported that MPA adversely affected the cartilage proteoglycan content of treated joints. These experiments involved multiple administrations of Depo-Medrol, resulting in the articular cartilage of the treated joints being exposed to relatively high concentrations of MPA\MP for extended periods. It is likely that under these circumstances the suppressive effects of MPA\MP on cellular metabolism would assume a greater relative importance over the anti-catabolic effects. This may explain why our findings differ substantially from these *in vivo* trials

This is the first study to confirm that high doses of Depo-Medrol have a direct toxic effect on equine chondrocytes. Interpretations of previous *in vivo* investigations (Chunekamrai *et al.*, 1989; Shoemaker *et al.*, 1992) have been complicated by coincidental proteoglycan losses, which may have also left the chondrocytes more susceptible to physical damage (Behrens *et al.*, 1976; McIlwraith & Vachon, 1988). Chondronecrosis, empty lacunae, intra-cartilaginous cysts, and deposition of hydroxyapatite have all been reported following intra-articular depot corticosteroid administrations in live animals (Behrens *et al.*, 1976; Ohira & Ishikawa, 1986; Chunekamrai *et al.*, 1989; Shoemaker *et al.*, 1992). The cytotoxic mechanism of action of corticosteroids for most tissues is thought to involve impairment of metabolic function (Haynes, 1991). The almost total inhibition of proteoglycan synthesis caused by the 2000  $\mu\text{g mL}^{-1}$  dose of MPA, and the increased percentage of cells with smaller nuclei and vacuolated cytoplasm, suggests this mechanism may also be important in equine articular cartilage. In equine joints, an increased incidence of degenerative cellular lesions has been associated with frequent administration of high doses of MPA (Chunekamrai *et al.*, 1989; Shoemaker *et al.*, 1992). Investigations involving lower

doses of MPA, and/or longer dose intervals, have tended to show fewer or no changes in chondrocyte viability (Marcoux, 1977; Trotter *et al.*, 1991). Thus, drug concentration, duration of exposure, and physical factors probably all determine how MPA affects chondrocyte viability *in vivo*.

Several *in vivo* studies have used the excipients of Depo-Medrol (water, Polyethylene glycol 3350, myristyl-gamma-picolinium chloride (MGP)) as a treatment control and showed no difference in either chondrocyte morphology or matrix composition from the controls (Marcoux, 1978; Myers & Stack, 1988; Chunekamrai *et al.*, 1989). However, Myers & Stack (1988) did find one of the excipients (MGP) caused a dose-dependant suppression of glycosaminoglycan synthesis when added at relatively high concentrations to *in vitro* cultures of canine articular cartilage. It is therefore possible that at the highest dose rate MGP may have also contributed to some of the suppressive effects seen.

Because Depo-Medrol suppressed proteoglycan synthesis in all treatment groups it cannot strictly be classified as a chondroprotective drug in the horse (as defined by Burkhardt & Ghosh, 1987). However, in many clinical situations the benefits associated with the anti-catabolic actions of Depo-Medrol could far outweigh the suppressive effect on proteoglycan synthesis. Assuming the anti-anabolic effects are not permanent (Fubini *et al.*, 1993), the proteoglycan content of articular cartilage is unlikely to be markedly affected by a single intra-articular administration of Depo-Medrol. Multiple short interval administrations would result in the articular cartilage within the joint being exposed to higher concentrations of MPA/MP for extended periods. Under these circumstances the anti-anabolic, and possibly even the cytotoxic, effects could assume greater importance. Also the administration of Depo-Medrol in joints with substantial pre-existing proteoglycan depletion, or cell death, might increase the time taken by the chondrocytes to replenish the proteoglycan content of these joints.

Phenylbutazone and other NSAIDs have been reported to interfere with cellular carbohydrate metabolism, and the synthesis of sulphated proteoglycans, in several species (Whitehouse & Haslam, 1962; Whitehouse, 1964; Bostrom *et al.*, 1964;

Palmoski & Brandt, 1979; Palmoski *et al.*, 1980; Palmoski & Brandt, 1983; Burkhardt & Ghosh, 1987). However, the literature is lacking in controlled reports of the effects of PBZ on equine articular cartilage. To our knowledge, this is the first reported investigation of the effects of PBZ on cultured explants of equine articular cartilage. The absence of any metabolic or cytotoxic effects at the lower concentrations (2-200  $\mu\text{g ml}^{-1}$ ) supports the *in vivo* findings of Denko (1964) (rat cartilage), but not the *in vitro* results of Whitehouse & Bostrum (1962) and Bostrum *et al.* (1964) (bovine cartilage). It is possible species variations in enzyme structure and affinity for PBZ may account for some of the differences.

Our results are consistent with PBZ being classified as a chondroprotective drug in the horse. At clinically applicable concentrations PBZ significantly reduced proteoglycan depletion, while having no effect on either proteoglycan synthesis or chondrocyte viability. Metabolic and cytotoxic effects were seen only at the 2000  $\mu\text{g ml}^{-1}$  concentration, which is at least 100-fold greater than concentrations achieved clinically in equine synovial fluid (Lehmann *et al.*, 1981; Lees *et al.*, 1987). It was concluded that the use of PBZ at clinically recommended dose rates was unlikely to impair directly the proteoglycan or cellular content of equine joints, while conferring some protective advantage against chondrocyte mediated catabolism.

This study demonstrates that the chondrocytes are quite capable of causing a substantial depletion of proteoglycans by themselves without any help from cytokines or proteolytic enzymes produced by synovial and migrant inflammatory cells. The increased proteoglycan catabolism may have resulted from increased enzyme synthesis, or activation of the substantial pool of latent enzymes already present in the matrix (Woessner & Selzer, 1984; Martel-Pelletier *et al.*, 1985; Flannery *et al.*, 1992). However, as the molecular mechanisms involved in inducing the synthesis and activation of proteolytic enzymes within articular cartilage are not fully understood (Flannery *et al.*, 1992; MacDonald *et al.*, 1992), we can only speculate about the means by which these two drugs reduced proteoglycan catabolism.

Most of the clinical effects of PBZ to date have been explained by its inhibition of the cyclo-oxygenase mediated production of prostaglandins (Vane, 1976; Lees & Higgins, 1985). However, at high concentrations, PBZ can also inhibit other enzymes including at least one matrix proteolytic enzyme (Whitehouse, 1962; Bostrum *et al.*, 1964; Burkhardt & Ghosh, 1987; May *et al.*, 1988; Meschter *et al.*, 1990). Equine chondrocytes subjected to adverse culture conditions have been shown to produce increased amounts of prostaglandin E<sub>2</sub> and the matrix proteolytic enzyme stromelysin (May *et al.*, 1991). The effects of increased prostaglandin production by equine chondrocytes are not known, but the addition of prostaglandins of the E series to articular cartilage cultures of other species has been shown to cause a proteoglycan depletion (Fulkerson *et al.*, 1979). Prostaglandins can affect both the rate of synthesis of new matrix proteolytic enzymes and the activation of the pool of latent enzymes already present in the matrix (Burkhardt & Ghosh, 1987). Thus, it is likely the chondroprotective effects of PBZ, like most of the clinical effects, may also predominantly result from inhibition of prostaglandin production.

Corticosteroids generally exert their effects through 'transcriptional regulation' resulting in an increased or decreased production of polypeptides (Haynes, 1991). These polypeptides can be enzymes, enzyme potentiators, enzyme inhibitors, or have one of many other effects on processes within the cell. Corticosteroids can also inhibit the production of inducible cyclo-oxygenase (COX-2) (Xie *et al.*, 1991; Kujubu *et al.*, 1991; O'Banion *et al.*, 1991). Increased synthesis of COX-2 is responsible for much of the sustained increases in prostaglandin production seen in many adversely stimulated tissues (Simmons *et al.*, 1993; Winn *et al.*, 1993). Inhibition of prostaglandin and or cytokine production by the chondrocytes may have been an important mechanism of action involved in the anti-catabolic effect of Depo-Medrol.

Thus, the anticatabolic mechanism of action of Depo-Medrol could have involved either direct or indirect suppression of new enzyme synthesis, or may have mainly involved inhibition of the activation of proteolytic enzymes already present in the matrix. However, as articular cartilage is thought to already contain a substantial pool of latent proteolytic enzymes (Flannery *et al.*, 1992), it is likely that much of the anti-catabolic

effect of Depo-Medrol resulted from a inhibition of the processes involved in the activation of these enzymes, rather than just a suppression of their synthesis. Inhibition of prostaglandin production could conceivably have been a common mechanism of action for both PBZ and Depo-Medrol. Further investigation is needed to define what mechanisms of action are involved in the anti-catabolic effects of these two drugs.

The histological observations generally agreed with the biochemical finding that the control group of explants for each drug was the most depleted in proteoglycan content. The large variability in cellular and matrix staining characteristics between explants further highlights the need for very large sample numbers for histological methods to demonstrate treatment differences reliably (Mankin, 1971; Hunziker *et al.*, 1992). Assessment of cell viability solely on nuclear morphology provided the most unequivocal measure of cell death. With the exception of the areas immediately adjacent to the cut edges of the explants, the distributions of proteoglycan loss and chondrocyte death were similar to those found in *in vivo* models of inflammatory joint disease (McIlwraith & Van Sickle, 1981; Gustafson *et al.*, 1992).

The small number of chondrocyte clusters seen in any one section supports the finding of others that the mitotic response of chondrocytes to insult is very limited (Hurtig *et al.*, 1988; Richardson & Clark, 1991). In contrast to the finding of Shoemaker *et al.* (1992) Depo-Medrol did not noticeably stimulate chondrocyte cluster formation. The appearance of the chondrocyte clusters found in the transitional and radiate zones is consistent with the finding of Poole *et al.* (1988) that the chondrocytes of these zones exist within fibrous capsules. The constraint of chondrocytes within felt-like pericellular capsules may help explain why damaged areas of articular cartilage are rarely repopulated, even though post-mitotic clusters of chondrocytes are often seen at their margins.

### C. SUMMARY & CONCLUSIONS

Methods were set up whereby explants of equine carpal articular cartilage could be successfully cultured for periods in excess of five days. The metabolic behaviour of the cultured explants resembled the *in vivo* response of articular cartilage to injury. All explants remained metabolically active and viable but suffered a net loss of proteoglycans. Proteoglycan loss was reduced by the presence of either phenylbutazone or Depo-Medrol. Depo-Medrol caused a dose-dependent suppression of proteoglycan synthesis at all concentrations, but chondrocyte viability was markedly inhibited only at doses well in excess of those used clinically. Phenylbutazone affected proteoglycan synthesis and cell viability at the very highest concentrations used only. At all concentrations, the anti-catabolic effects of each drug influenced the proteoglycan content of the explants far more than did any anti-anabolic or cytotoxic drug effect.

The results suggest that the therapeutic potential of both phenylbutazone and Depo-Medrol may not be just restricted to their anti-inflammatory effects on the soft tissues of the joint, but may also involve a suppression of the synthesis and/or activation of proteolytic enzymes within the cartilage itself. Furthermore, the results cast some doubt over whether clinically recommended doses of either of these drugs substantially contribute to the progression of degenerative joint disease through any direct negative effect on the chondrocytes.

However, the extrapolation of *in vitro* results to clinical situations has always been somewhat tenuous, especially when only a limited number of parameters are measured. This study examined only quantitative differences in the proteoglycan content of the matrix of cultured explants of equine articular cartilage. Changes in the functional capacity of the restraining collagen network were not looked for, nor were any qualitative changes in the proteoglycans themselves. Never-the-less, *in vitro* studies can improve the understanding of the mechanisms of drug action and their relationship to concentration. This information, when combined with an understanding of the pathogenesis of the condition being treated, can lead to more rational drug use.

The clinical application of these results is also limited because the study examined only the short term, dose related effects of a MPA formulation on chondrocytes, isolated from other physiological and physical influences. No attempt was made to determine how much of the MPA was converted into the active MP form. Continued investigation is required to assess the concentration related effects of MP alone, and to determine the duration of its suppressive effects on proteoglycan synthesis. Cartilage destruction associated with inflammatory joint disease may also involve chondrocytes being influenced by cytokines produced by other cell types, as well as proteolytic enzymes released from the synoviocytes and migrant inflammatory cells. Further research is needed to define what effects

- (a) IL-1, TNF, and PGE<sub>2</sub> have on proteoglycan synthesis and catabolism.
- (b) MP and PBZ may have on the effects of IL-1, TNF, and PGE<sub>2</sub>.
- (c) MP and PBZ have on the release of cytokines and proteolytic enzymes from cultures of equine synovium, neutrophils and monocytes.

Future investigations should concentrate on clinically applicable concentrations of MP, PBZ, and cytokines and perhaps could also investigate whether combinations of MP and PBZ may have an additive or synergistic effect.

## APPENDIX

## A. TABLES

$\mu\text{g}$	Galactosamine	Glucosamine	Chondroitin $\text{SO}_4$
2.5	0.071	0.079	0.072
5	0.139	0.149	0.123
10	0.262	0.287	0.219
15	0.398	0.420	0.331
20	0.532	0.540	0.432

Table 1. Mean absorbances at 524 nm of three sets of standard solutions assayed for their amino sugar content.

$\mu\text{g}$ Amino sugar	0.5 hours	2 hours	23 hours
<b>Glucosamine</b>			
5	0.146	0.131	0.090
10	0.266	0.239	0.162
15	0.418	0.377	0.243
20	0.554	0.503	0.315
<b>Galactosamine</b>			
5	0.151	0.130	0.076
10	0.284	0.255	0.156
15	0.391	0.348	0.219
20	0.516	0.462	0.274
<b>Chondroitin sulphate</b>			
5	0.119	0.107	0.068
10	0.218	0.202	0.124
15	0.295	0.272	0.165
20	0.392	0.362	0.210

Table 2. Effect of time on the amino sugar assay (absorbances at 524 nm)

Standard	cpm	dpm	CE	H number
A	191312	201770	0.948	57
B	183871	201770	0.911	128
C	167051	201770	0.828	214
D	146316	201770	0.725	282
E	133324	201770	0.661	312
F	110333	201770	0.547	350

**Table 3.**  $^{14}\text{C}$  Quench standards

Washes	Live chondrocytes	Dead chondrocytes
0	10653 $\pm$ 4525	6393 $\pm$ 1471
2	3938 $\pm$ 1072	162 $\pm$ 172
3	3582 $\pm$ 1033	80 $\pm$ 100

**Table 4.** Effect of two different wash techniques on biologically bound and non-bound  $^{35}\text{S}$  content. (mean dpm  $\text{mg}^{-1}$ )

DNA (ng)	Rep 1 (fluorescence)	Rep 2 (fluorescence)	Rep 3 (fluorescence)	Mean
Blank	0.00	0.01	0.02	0.01
100	0.13	0.14	0.16	0.14
200	0.30	0.30	0.30	0.30
300	0.47	0.46	0.44	0.46
400	0.61	0.60	0.55	0.59
500	0.77	0.71	0.70	0.73

**Table 5.** Assay of calf thymus DNA standards (ng DNA versus fluorescence).

Galactosamine ( $\mu\text{g}$ )	Rep 1	Rep 2	Mean
3.75	0.050	0.060	0.055
7.50	0.135	0.140	0.138
15.0	0.230	0.265	0.248
22.5	0.360	0.385	0.373

Table 6. Absorbances from chondroitin sulphate standards in water, dialysed and oven digested.

Galactosamine ( $\mu\text{g}$ )	Rep 1	Rep 2	Mean
3.75	0.040	0.080	0.060
7.50	0.105	0.160	0.133
15.0	0.170	0.220	0.195
22.5	0.415	0.415	0.410

Table 7. Absorbances from chondroitin sulphate standards in DMEM, dialysed and oven digested.

Day	Explants (n)	Mean AS ( $\mu\text{g}$ )	Mean Weight (mg)	AS Weight <sup>-1</sup> $\pm$ SD ( $\mu\text{g mg}^{-1}$ )	CV (%)
0	10	79.8	3.59	22.5 $\pm$ 5.4	24
1	10	75.8	4.11	18.2 $\pm$ 3.7	20
2	10	76.2	4.12	18.4 $\pm$ 4.1	22
3	10	58.8	3.71	15.8 $\pm$ 1.9	12
4	10	52.6	3.81	13.8 $\pm$ 3.3	24
5	10	63.4	3.82	16.5 $\pm$ 2.6	16
6	9	61.7	3.74	16.5 $\pm$ 2.4	14
7	10	55.0	3.53	15.5 $\pm$ 3.3	21
mean			3.80		19

Table 8. Effect of culture duration on the amino sugar content (AS) of cartilage explants relative to their initial wet weight.

Day	Explants (n)	Mean AS ( $\mu\text{g}$ )	Mean DNA ( $\mu\text{g}$ )	AS DNA <sup>-1</sup> $\pm$ SD ( $\mu\text{g } \mu\text{g}^{-1}$ )	CV (%)
0	10	79.8	2.71	29.7 $\pm$ 5.9	20
1	10	75.8	3.08	24.8 $\pm$ 7.2	29
2	10	76.2	2.82	26.9 $\pm$ 7.8	29
3	10	58.8	2.92	20.4 $\pm$ 4.3	21
4	10	52.6	2.14	25.5 $\pm$ 8.7	34
5	10	63.4	2.21	28.8 $\pm$ 5.8	20
6	9	61.7	2.54	24.4 $\pm$ 5.4	22
7	10	55.0	2.18	26.0 $\pm$ 8.8	35
mean			2.58		26

Table 9. Effect of culture duration on the amino sugar content (AS) of cartilage explants relative to their final DNA content.

Day	Explants (n)	Mean dpm	Mean Weight (mg)	dpm Weight <sup>-1</sup> $\pm$ SD (dpm mg <sup>-1</sup> )	CV (%)
0	10	21	3.59	6 $\pm$ 5	83
1	10	2159	4.11	525 $\pm$ 176	34
2	10	4414	4.12	1074 $\pm$ 345	32
3	10	5655	3.71	1520 $\pm$ 796	52
4	10	5384	3.81	1466 $\pm$ 786	54
5	10	5515	3.82	1435 $\pm$ 512	36
mean			3.86		* 42

\* mean excludes day 0 C.V. %

Table 10. Effect of culture duration on the incorporation of <sup>35</sup>S by cartilage explants relative to their initial wet weight.

Day	Explants (n)	Mean dpm	Mean DNA ( $\mu\text{g}$ )	dpm DNA <sup>-1</sup> $\pm$ SD (dpm $\mu\text{g}^{-1}$ )	CV (%)
0	10	21	2.7	7 $\pm$ 6	76
1	10	2159	3.1	700 $\pm$ 260	37
2	10	4414	2.8	1552 $\pm$ 451	29
3	10	5655	2.9	1860 $\pm$ 879	47
4	10	5384	2.1	2546 $\pm$ 1058	42
5	10	5515	2.2	2529 $\pm$ 1016	40
mean			2.6		* 39

\* mean excludes day 0 C.V. %

**Table 11.** Effect of culture duration on the incorporation of <sup>35</sup>S by cartilage explants relative to their final DNA content.

Treatment group	Explants (n)	Mean % control ± SEM *
Control	61	<sup>A</sup> 100.00 ± 3.28
20 µg mL <sup>-1</sup>	67	<sup>B</sup> 108.12 ± 3.04
200 µg mL <sup>-1</sup>	69	<sup>C</sup> 106.54 ± 3.36
2000 µg mL <sup>-1</sup>	66	<sup>D</sup> 113.31 ± 2.32

**Table 12.** The dose related effect of a methylprednisolone acetate formulation on the amino sugar content of cultured explants of equine middle carpal joint articular cartilage.

\* The amino sugar mg<sup>-1</sup> value of each explant was divided by the mean amino sugar mg<sup>-1</sup> value of the control group from the same horse. All results were then combined into four control/treatment groups, averaged and expressed as percentages.

When B, C, and D are combined they are significantly different from A ( $p = 0.017$ ). When just B and C are combined they are not significantly different from A ( $p = 0.075$ ). D is not significantly different from groups B and C combined.

Treatment group	Explants (n)	Mean % control ± SEM *	Square root transformation ± SEM **
Control	61	99.99 ± 4.92	<sup>A</sup> 0.9836 ± 0.2320
20 µg mL <sup>-1</sup>	67	76.15 ± 3.64	<sup>B</sup> 0.8543 ± 0.0219
200 µg mL <sup>-1</sup>	69	50.06 ± 3.42	<sup>C</sup> 0.6800 ± 0.0237
2000 µg mL <sup>-1</sup>	66	5.54 ± 0.85	<sup>D</sup> 0.2035 ± 0.0151

**Table 13.** The dose related effect of a methylprednisolone acetate formulation on the incorporation of <sup>35</sup>S by cultured explants of equine middle carpal joint articular cartilage.

\* The dpm mg<sup>-1</sup> value of each explant was divided by the mean dpm mg<sup>-1</sup> value of the control group from the same horse. All results were then combined into four control/treatment groups, averaged and expressed as percentages.

\*\* The dpm mg<sup>-1</sup> value of each explant was divided by the mean dpm mg<sup>-1</sup> value of the control group from the same horse. The square root of each result was then calculated before all the results were combined into the four control/treatment groups and averaged.

When B, C, and D are combined they are significantly different from A ( $p < 0.001$ ). Group A is also significantly different from groups B and C combined ( $p < 0.001$ ). Groups B and C combined are significantly different from group D ( $p < 0.001$ ).

Treatment group	Explants (n)	Cells(%) dead Mean $\pm$ SD	Cells(%) dying during culture * Mean $\pm$ SD	Log (Cells(%) dying during culture + 1) Mean $\pm$ SD
Not cultured	35	1.1 $\pm$ 1.3		
Control	35	2.9 $\pm$ 2.7	1.9 $\pm$ 2.2	0.360 $\pm$ 0.299
20 $\mu\text{g mL}^{-1}$	36	1.9 $\pm$ 1.4	1.1 $\pm$ 1.3	0.255 $\pm$ 0.238
200 $\mu\text{g mL}^{-1}$	36	2.9 $\pm$ 3.0	1.9 $\pm$ 2.2	0.356 $\pm$ 0.298
2000 $\mu\text{g mL}^{-1}$	36	12.6 $\pm$ 9.4	11.5 $\pm$ 9.0	0.960 $\pm$ 0.385

**Table 14.** The dose related effect of a methylprednisolone acetate formulation on the viability of chondrocytes within cultured explants of equine middle carpal joint articular cartilage.

\* The percentage of chondrocytes determined as dead in each cultured explant was reduced by the average percentage of chondrocytes found dead in the non-cultured group of explants from the same horse. Any resulting negative percentages were rounded up to zero, then all results were combined into the four control/treatment groups and averaged.

The result from the 2000  $\mu\text{g mL}^{-1}$  group was the only one significantly different from the others ( $p < 0.001$ ).

Treatment group	Explants (n <sub>i</sub> )	Mean % of control ± SEM *
Control	62	<sup>A</sup> 100.00 ± 2.92
2 µg mL <sup>-1</sup>	61	<sup>B</sup> 106.46 ± 2.81
20 µg mL <sup>-1</sup>	62	<sup>C</sup> 111.32 ± 3.95
200 µg mL <sup>-1</sup>	60	<sup>D</sup> 108.67 ± 3.75
2000 µg mL <sup>-1</sup>	36	<sup>E</sup> 113.35 ± 4.79

**Table 15.** The effect of different concentrations of phenylbutazone on the amino sugar content of cultured explants of equine middle carpal joint articular cartilage.

\* The amino sugar mg<sup>-1</sup> value of each explant was divided by the mean amino sugar mg<sup>-1</sup> value of the control group from the same horse. All results were then combined into five control/treatment groups, averaged and expressed as percentages.

Groups B, C, D, and E combined are significantly different from group A ( $p = 0.011$ ). A is also significantly different from groups B and C combined ( $p < 0.001$ ). Groups B and C combined are not significantly different from groups D and E combined ( $p = 0.59$ ).

Treatment group	Explants (n)	Mean % control ± SEM *	Square root transformation ± SEM **
Control	62	99.99 ± 3.60	0.9905 ± 0.0176
2 µg mL <sup>-1</sup>	61	103.09 ± 3.18	1.0080 ± 0.0157
20 µg mL <sup>-1</sup>	62	92.54 ± 3.02	0.9536 ± 0.0160
200 µg mL <sup>-1</sup>	60	98.29 ± 3.32	0.9826 ± 0.0172
2000 µg mL <sup>-1</sup>	36	5.13 ± 0.14	0.0057 ± 0.0073

**Table 16.** The effect of different concentrations of PBZ on the incorporation of <sup>35</sup>S by cultured explants of equine middle carpal joint articular cartilage.

\* The dpm mg<sup>-1</sup> value of each explant was divided by the mean dpm mg<sup>-1</sup> value of the control group from the same horse. All results were then combined into five control/treatment groups, averaged and expressed as percentages.

\*\* The dpm mg<sup>-1</sup> value of each explant was divided by the mean dpm mg<sup>-1</sup> value of the control group from the same horse. The square root of each result was then calculated before all the results were combined into the five control/treatment groups and averaged.

The result from the 2000 µg mL<sup>-1</sup> group was the only one significantly different from the others (p < 0.001).

Treatment group	Explants (n)	Cells(%) dead Mean $\pm$ SD	Cells(%) dying during culture Mean $\pm$ SD ****	Log (Cells(%) dying during culture + 1) Mean $\pm$ SD
Not cultured	29 *	1.0 $\pm$ 1.1		
Control	32	2.2 $\pm$ 2.4	1.6 $\pm$ 2.2	0.302 $\pm$ 0.295
2 $\mu\text{g mL}^{-1}$	32	2.9 $\pm$ 3.1	2.3 $\pm$ 3.1	0.393 $\pm$ 0.324
20 $\mu\text{g mL}^{-1}$	32	2.1 $\pm$ 1.9	1.5 $\pm$ 1.9	0.316 $\pm$ 0.262
200 $\mu\text{g mL}^{-1}$	31 **	1.8 $\pm$ 2.1	1.3 $\pm$ 2.3	0.251 $\pm$ 0.288
2000 $\mu\text{g mL}^{-1}$	28 ***	9.8 $\pm$ 4.8	9.8 $\pm$ 5.1	0.913 $\pm$ 0.336

**Table 17.** The effect of different concentrations of phenylbutazone on the viability of chondrocytes within cultured explants of equine middle carpal joint articular cartilage.

\* Non-cultured explants were not kept from one horse, and four out of the five other horses had six explants kept.

\*\* One explant went missing during processing.

\*\*\* Only four explants instead of five were cultured from four of the six horses.

\*\*\*\* The percentage of chondrocytes determined as dead in each cultured explant was reduced by the average percentage of chondrocytes found dead in the non-cultured group of explants from the same horse. Any resulting negative percentages were rounded up to zero, then all results were combined into the five control/treatment groups and averaged.

The result from the 2000  $\mu\text{g mL}^{-1}$  was the only one significantly different from the others ( $p < 0.001$ ).

## B. STATISTICAL ANALYSES

Method of analysis = Linear contrasts

### Depo-Medrol trial

#### 1. Effect of Depo-Medrol on amino sugar content

First null hypothesis:  $U_{\text{control}} = U_{\text{treatments}}$  ( $U_i$  = Population mean)

$$\text{Contrast} = \hat{C} = c_1 Y_{\text{control}} + c_2 Y_{20\mu\text{g mL}^{-1}} + c_3 Y_{200\mu\text{g mL}^{-1}} + c_4 Y_{2000\mu\text{g mL}^{-1}}$$

$$\text{Where } \sum c_i = 0$$

$Y_i$  = sample mean of the control or treatment groups

#### Relevant data from analysis of variance

$$p = 0.0455$$

$$MS_{\text{error}} = 0.07055$$

$$\text{Bartlett's } X^2 = 1.26, p = 0.7386$$

$$Y_{\text{control}} = 1.0000, \quad n_1 = 61$$

$$Y_{20\mu\text{g mL}^{-1}} = 1.0812, \quad n_2 = 67$$

$$Y_{200\mu\text{g mL}^{-1}} = 1.0654, \quad n_3 = 69$$

$$Y_{2000\mu\text{g mL}^{-1}} = 1.1331, \quad n_4 = 66$$

$$\begin{aligned} \text{Contrast, } \hat{C} &= 3Y_{\text{control}} - Y_{20\mu\text{g mL}^{-1}} - Y_{200\mu\text{g mL}^{-1}} - Y_{2000\mu\text{g mL}^{-1}} \\ &= -0.2797 \end{aligned}$$

$$\begin{aligned} SS_{\text{contrast}} &= \frac{(\hat{C})^2}{\sum \frac{(c_i)^2}{n_i}} = \frac{(-0.2797)^2}{\frac{(3)^2}{61} + \frac{(-1)^2}{67} + \frac{(-1)^2}{69} + \frac{(-1)^2}{66}} \\ &= 0.4072 \end{aligned}$$

$$F_{\text{ratio}} = \frac{SS_{\text{error}}}{MS_{\text{error}}} = \frac{0.4072}{0.07055} = 5.772$$

$$F_{(1, 259)}, p = 0.01699$$

$$\text{Second null hypothesis: } U_{\text{control}} = \frac{U_{20\mu\text{g mL}^{-1}} + U_{200\mu\text{g mL}^{-1}}}{2}$$

$$\begin{aligned} \text{Contrast} &= 2Y_{\text{control}} - 1Y_{20\mu\text{g mL}^{-1}} - 1Y_{200\mu\text{g mL}^{-1}} - 0Y_{2000\mu\text{g mL}^{-1}} \\ &= 0.1466 \end{aligned}$$

$$SS_{\text{contrast}} = 0.2262$$

$$F_{\text{ratio}} = 3.207$$

$$F_{(1,195)}, p = 0.0749$$

$$\text{Third null hypothesis: } = \frac{U_{20\mu\text{g mL}^{-1}} + U_{200\mu\text{g mL}^{-1}}}{2} = U_{2000\mu\text{g mL}^{-1}}$$

$$\begin{aligned} \text{Contrast} &= 0Y_{\text{control}} - 1Y_{20\mu\text{g mL}^{-1}} - 1Y_{200\mu\text{g mL}^{-1}} + 2Y_{2000\mu\text{g mL}^{-1}} \\ &= 0.1196 \end{aligned}$$

$$SS_{\text{contrast}} = 0.1589$$

$$F_{\text{ratio}} = 2.2522$$

$$F_{(1,200)}, p = 0.1350$$

## 2. Effect of Depo-Medrol on $^{35}\text{SO}_4$ incorporation

Relevant data from analysis of variance

(a) For raw data

$$p = 0.0000$$

$$MS_{\text{error}} = 0.07889$$

$$\text{Bartlett's } X^2 = 136.04, p = 0.0000$$

$$Y_{\text{control}} = 0.9999, \quad n_1 = 61$$

$$Y_{20\mu\text{g mL}^{-1}} = 0.7615, \quad n_2 = 67$$

$$Y_{200\mu\text{g mL}^{-1}} = 0.5006, \quad n_3 = 69$$

$$Y_{2000\mu\text{g mL}^{-1}} = 0.0554, \quad n_4 = 66 \quad (n-k) = 259$$

**(b) For square root transformation (sqrt) data**

$$p = 0.0000$$

$$MS_{\text{error}} = 0.02977$$

$$\text{Bartlett's } X^2 = 15.25, p = 0.0016$$

$$Y_{\text{control}} = 0.9836, n_1 = 61$$

$$Y_{20\mu\text{g mL}^{-1}} = 0.8543, n_2 = 67$$

$$Y_{200\mu\text{g mL}^{-1}} = 0.6800, n_3 = 69$$

$$Y_{2000\mu\text{g mL}^{-1}} = 0.2035, n_4 = 66 \quad (n-k) = 259$$

**(c) For log transformed data**

$$p = 0.0000$$

$$MS_{\text{error}} = 0.10628$$

$$\text{Bartlett's } X^2 = 108.95, p = 0.0000$$

$$Y_{\text{control}} = 3.9718, n_1 = 61$$

$$Y_{20\mu\text{g mL}^{-1}} = 3.8417, n_2 = 67$$

$$Y_{200\mu\text{g mL}^{-1}} = 3.6247, n_3 = 69$$

$$Y_{2000\mu\text{g mL}^{-1}} = 2.4672, n_4 = 66 \quad (n-k) = 259$$

The equal variance assumption is most critical when sample sizes are markedly different. The sqrt transformation results in the lowest  $X^2$  value for Bartlett's test. The variance associated with the high treatment group is primarily responsible for violating the assumption of equal variances as its mean approaches zero. The differences between the comparisons are so great that the violation of the assumption of equal variances is highly unlikely to affect the results at the level they are to be interpreted.

First null hypothesis:  $U_{\text{control}} = U_{\text{treatments}}$

Using the sqrt transformed data

$$\begin{aligned} \hat{\text{Contrast}} &= 4Y_{\text{control}} + 1Y_{20\mu\text{g mL}^{-1}} + 1Y_{200\mu\text{g mL}^{-1}} + 1Y_{2000\mu\text{g mL}^{-1}} \\ &= 1.213 \\ SS_{\text{contrast}} &= 7.66 \\ F_{\text{statistic}} &= 257.4 \\ F_{(1,259)}, p &= 0.000 \end{aligned}$$

Second null hypothesis  $U_{\text{control}} = \frac{U_{20\mu\text{g mL}^{-1}} + U_{200\mu\text{g mL}^{-1}}}{2}$

$$\begin{aligned} \hat{\text{Contrast}} &= 2Y_{\text{control}} - 1Y_{20\mu\text{g mL}^{-1}} - 1Y_{200\mu\text{g mL}^{-1}} - 0Y_{2000\mu\text{g mL}^{-1}} \\ &= 0.4329 \\ SS_{\text{contrast}} &= 1.9728 \\ F_{\text{ratio}} &= 66.27 \\ F_{(1, 195)}, p &= 0.000 \end{aligned}$$

Third null hypothesis  $\frac{U_{20\mu\text{g mL}^{-1}} + U_{200\mu\text{g mL}^{-1}}}{2} = U_{2000\mu\text{g mL}^{-1}}$

$$\begin{aligned} \hat{\text{Contrast}} &= 0Y_{\text{control}} + 1Y_{20\mu\text{g mL}^{-1}} + 1Y_{200\mu\text{g mL}^{-1}} - 2Y_{2000\mu\text{g mL}^{-1}} \\ &= 1.1273 \\ SS_{\text{contrast}} &= 14.118 \\ F_{\text{ratio}} &= 474.25 \\ F_{(1, 200)}, p &= 0.0000 \end{aligned}$$

## Phenylbutazone trial

### 1. Effect of phenylbutazone on amino sugar content

First null hypothesis:  $U_{\text{control}} = U_{\text{treatments}}$

$$\hat{C} = 4Y_{\text{control}} - 1Y_{2\mu\text{g mL}^{-1}} - 1Y_{20\mu\text{g mL}^{-1}} - 1Y_{200\mu\text{g mL}^{-1}} - 1Y_{2000\mu\text{g mL}^{-1}}$$

Relevant data from analysis of variance

(a) For raw data

$$p = 0.0933$$

$$MS_{\text{error}} = 0.07123$$

$$\text{Bartlett's } X^2 = 10.81, p = 0.0288$$

$Y_{\text{control}}$	= 1.0000,	$n_1 = 62$	
$Y_{2\mu\text{g mL}^{-1}}$	= 1.0646,	$n_2 = 61$	
$Y_{20\mu\text{g mL}^{-1}}$	= 1.1132,	$n_3 = 62$	
$Y_{200\mu\text{g mL}^{-1}}$	= 1.0867,	$n_4 = 60$	
$Y_{2000\mu\text{g mL}^{-1}}$	= 1.1335,	$n_5 = 36$	$(n-k) = 276$

$$\begin{aligned} \hat{\text{Contrast}} &= 4Y_{\text{control}} - Y_{2\mu\text{g mL}^{-1}} - Y_{20\mu\text{g mL}^{-1}} - Y_{200\mu\text{g mL}^{-1}} - Y_{2000\mu\text{g mL}^{-1}} \\ &= -0.3980 \end{aligned}$$

$$SS_{\text{contrast}} = 0.47281$$

$$F_{\text{ratio}} = 6.6377$$

$$F_{(1, 276)}, p = 0.01050$$

**(b) For square root transformation data**

$$p = 0.1341$$

$$MS_{\text{error}} = 0.01722$$

$$\text{Bartlett's } X^2 = 9.59, p = 0.0478$$

$$\begin{aligned} Y_{\text{control}} &= 0.9933, & n_1 &= 62 \\ Y_{2\mu\text{g mL}^{-1}} &= 1.0262, & n_2 &= 61 \\ Y_{20\mu\text{g mL}^{-1}} &= 1.0439, & n_3 &= 62 \\ Y_{200\mu\text{g mL}^{-1}} &= 1.0331, & n_4 &= 60 \\ Y_{2000\mu\text{g mL}^{-1}} &= 1.0567, & n_5 &= 36 \end{aligned} \quad (n-k) = 276$$

$$\begin{aligned} \hat{\text{Contrast}} &= 4Y_{\text{control}} - Y_{2\mu\text{g mL}^{-1}} - Y_{20\mu\text{g mL}^{-1}} - Y_{200\mu\text{g mL}^{-1}} - Y_{2000\mu\text{g mL}^{-1}} \\ &= -0.1867 \end{aligned}$$

$$SS_{\text{contrast}} = 0.104$$

$$F_{\text{ratio}} = 6.04$$

$$F_{(1, 276)}, p = 0.01460$$

$$\text{Second null hypothesis } U_{\text{control}} = \frac{U_{2\mu\text{g mL}^{-1}} + U_{20\mu\text{g mL}^{-1}}}{2}$$

$$\hat{C} = 2Y_{\text{control}} - 1Y_{2\mu\text{g mL}^{-1}} - 1Y_{20\mu\text{g mL}^{-1}} - 0Y_{200\mu\text{g mL}^{-1}} - 0Y_{2000\mu\text{g mL}^{-1}}$$

**(a) Non-transformed data**

$$\text{Contrast} = -0.1778$$

$$SS_{\text{contrast}} = 0.4880$$

$$F_{\text{ratio}} = 6.846$$

$$F_{(1, 195)}, p = 0.00964$$

**(b) Sqrt transformation data**

$$\text{Contrast} = -0.0835$$

$$SS_{\text{contrast}} = 0.07185$$

$$F_{\text{ratio}} = 4.17$$

$$F_{(1, 195)}, p = 0.04210$$

$$\text{Second null hypothesis} = U_{2\mu\text{g mL}^{-1}} + U_{20\mu\text{g mL}^{-1}} = U_{200\mu\text{g mL}^{-1}} + U_{2000\mu\text{g mL}^{-1}}$$

$$\hat{C} = 0Y_{\text{control}} + 1Y_{2\mu\text{g mL}^{-1}} + 1Y_{20\mu\text{g mL}^{-1}} - 1Y_{200\mu\text{g mL}^{-1}} - 1Y_{2000\mu\text{g mL}^{-1}}$$

**(a) Non-transformed data**

$$\begin{aligned} \text{Contrast} &= -0.0424 \\ \text{SS}_{\text{contrast}} &= 0.02336 \\ \text{F}_{\text{ratio}} &= 0.3238 \\ \text{F}_{(1, 209)}, \text{ p} &= 0.56995 \end{aligned}$$

**(b) Sqrt transformation data**

$$\begin{aligned} \text{Contrast} &= -0.0197 \\ \text{SS}_{\text{contrast}} &= 0.00504 \\ \text{F}_{\text{ratio}} &= 0.2928 \\ \text{F}_{(1, 209)}, \text{ p} &= 0.58902 \end{aligned}$$

**2. Effect of phenylbutazone on  $^{35}\text{SO}_4$  incorporation**

$$\text{First null hypothesis: } U_{\text{control}} = U_{\text{treatments}}$$

$$\hat{C} = 4Y_{\text{control}} - 1Y_{2\mu\text{g mL}^{-1}} - 1Y_{20\mu\text{g mL}^{-1}} - 1Y_{200\mu\text{g mL}^{-1}} - 1Y_{2000\mu\text{g mL}^{-1}}$$

**(a) For raw data**

$$p = 0.0000$$

$$\text{MS}_{\text{error}} = 0.05807$$

$$\text{Bartlett's } X^2 = 200.84, p = 0.0000$$

$$Y_{\text{control}} = 0.9999, \quad n_1 = 62$$

$$Y_{2\mu\text{g mL}^{-1}} = 1.0309, \quad n_2 = 61$$

$$Y_{20\mu\text{g mL}^{-1}} = 0.9254, \quad n_3 = 62$$

$$Y_{200\mu\text{g mL}^{-1}} = 0.9829, \quad n_4 = 60 \quad (n-k) = 276$$

$$Y_{2000\mu\text{g mL}^{-1}} = 0.0051, \quad n_5 = 36$$

**(b) For square root transformation data**

$$p = 0.0000$$

$$MS_{\text{error}} = 0.01513$$

$$\text{Bartlett's } X^2 = 44.19, p = 0.0000$$

$$\begin{aligned} Y_{\text{control}} &= 0.9905, & n_1 &= 62 \\ Y_{2\mu\text{g mL}^{-1}} &= 1.0803, & n_2 &= 61 \\ Y_{20\mu\text{g mL}^{-1}} &= 0.9536, & n_3 &= 62 \\ Y_{200\mu\text{g mL}^{-1}} &= 0.9828, & n_4 &= 60 \quad (n-k) = 276 \\ Y_{2000\mu\text{g mL}^{-1}} &= 0.0572, & n_5 &= 36 \end{aligned}$$

**(c) For log transformed data**

$$p = 0.0000$$

$$MS_{\text{error}} = 0.10628$$

$$\text{Bartlett's } X^2 = 108.95, p = 0.0000$$

$$\begin{aligned} Y_{\text{control}} &= 3.9832, & n_1 &= 62 \\ Y_{2\mu\text{g mL}^{-1}} &= 4.0005, & n_2 &= 61 \\ Y_{20\mu\text{g mL}^{-1}} &= 3.9508, & n_3 &= 62 \\ Y_{200\mu\text{g mL}^{-1}} &= 3.9765, & n_4 &= 60 \quad (n-k) = 276 \\ Y_{2000\mu\text{g mL}^{-1}} &= 1.3306, & n_5 &= 36 \end{aligned}$$

**NB:** None of the transformations result in means with similar variances. The equal variance assumption is most critical when sample sizes are markedly different. The sqrt transformation results in the lowest  $X^2$  value for Bartlett's test. The variance associated with the extra treatment group, whose mean approaches zero, is primarily responsible for violating the assumption of equal variances. Only the extra groups mean is markedly different from the other means. The differences between the extra groups mean and the other groups means are so great that the violation of the assumption of equal variances is considered not to affect the results at the level they are to be interpreted at.

**Conclusion 1:** Phenylbutazone can effect the incorporation of radiolabel into tissue cultured explants of equine articular cartilage.

**Conclusion 2:** The effect is not statistically significant at concentrations achieved clinically in equine joints.

**Conclusion 3:** The effect is greatest at higher concentrations.

**Note:** These would be the conclusions reached if the data are analyzed in the same way as the methylprednisolone data. However, examination of the data clearly shows very little effect in any group other than the extra group, where the effect is dramatic.

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