THE EFFECT OF MATRIX DEPLETION ON ARTICULAR CARTILAGE.

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The object of the present study was to investigate the effect of matrix depletion on the morphology, ultrastructure and metabolism of adult bovine cartilage in explant culture. Matrix depletion was achieved by incubating the tissue in medium containing either Streptomyces hyaluronidase (used at 10 unit.ml⁻¹) or Clostridium collagenase (used at either 20 or 100 unit.ml⁻¹) for 24 hr at 37°C. The tissue was subsequently cultured for a further two weeks in medium without enzymes. Control explants were cultured in medium without enzymes throughout the culture period. The morphology and ultrastructure of matrix-depleted tissue has been investigated using light and both transmission and scanning electron microscopy. DNA synthesis has been assessed using tritiated thymidine labelling, whilst proteoglycan (PG) synthesis has been investigated using ³⁵S labelling and immunolocalisation.

Proteoglycans and hyaluronan were lost from control tissue at a steady rate, equating to about 9% per day for PG and 5% per day for hyaluronan. An increased loss of PG and hyaluronan was detected from both hyaluronidase and collagenase-treated tissue during enzyme incubation although the rate of loss rapidly returned to normal once the enzymes were removed. The loss of collagen from control and hyaluronidase-treated cultures was minimal, whilst collagenase-treated cultures lost up to 50% of their collagen during treatment. The rate of PG synthesis in control and hyaluronidase-treated explants showed an initial rise followed by a reduction throughout the rest of the culture period. Collagenase-treated explants exhibited an increased rate of PG synthesis.

We have reported that PG depletion in hyaluronidase-treated cultures is primarily from the surface zones and that the overall ultrastructure and architecture remains intact. Collagenase-induced disruption of tissue architecture has been reported and is most noted in the surface zones. Cellular changes and the formation of abnormal outgrowths have been described in collagenase-treated tissue.

The investigation of DNA synthesis, as assessed by tritiated thymidine labelling, revealed that few chondrocytes had undergone DNA replication during the culture period in control and hyaluronidase-treated cartilage (about 4% in two weeks). We have detected an increase in the number of labelled cells in collagenase-treated tissue. The increase was dose-dependent and most marked in area of the tissue which showed the greatest morphological tissue disruption. A link between cell flattening and the onset of DNA synthesis is proposed, based on observations of explant cultures and cultures of isolated chondrocytes.

We have reported the presence of novel chondroitin sulphate epitopes, recognised by the antibodies 3B3 (without chondroitinase) and 7D4 in both control and enzyme-treated cartilage from day 3 onwards. These epitopes are not normally found in normal adult cartilage and showed differential distribution in cultured cartilage.
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GENERAL INTRODUCTION
General Introduction

Adult articular cartilage is a specialised connective tissue which covers the articulating surfaces of bones and is vital for the correct functioning of joints. In general, three main functions are performed by normal articular cartilage in synovial joints (Kempson, 1979). First, it prevents abrasive interactions occurring between adjacent bones and secondly, it prevents high stresses from being transmitted to the bone below (Weightman & Kempson, 1979). Paul (1967) has shown that stresses may reach four times body weight during normal walking and are potentially damaging to the bone (Swanson et al., 1971; Freeman et al., 1975). Articular cartilage reduces stress by increasing the contact area of articulating surfaces and by evening the load applied at the subchondral bone interface (Day et al., 1975). The third main function of articular cartilage is to provide highly lubricated, almost frictionless surfaces to aid articulation and prevent wear (Swanson, 1979; Radin et al., 1970).

Development of articular cartilage.

The development of articular cartilage is poorly understood, but is believed to occur in an integrated fashion with the overall formation of skeletal elements and joints. Whilst the majority of studies on skeletal development have utilised the chick embryo as a model, the overall pattern appears to be similar in most higher vertebrates. In general the development of bony elements is preceded by the formation of a precartilaginous cell condensation within the mesenchyme (Fell, 1925; Thorogood & Hinchliffe, 1975; Ede, 1983). This appears to be due to cell movement (Ede, 1981; Ede & Wilby, 1981) rather than an increase in cell division (Janners & Searls, 1970). Soon after condensation formation, the cells start to produce a cartilaginous matrix containing type II collagen (Linsenmayer et al., 1973) and cartilage proteoglycan (Searls, 1965; Vasan & Lash, 1979). The developing rudiment grows by a combination of cell division, cell enlargement and matrix secretion (Hinchliffe, 1983; Rooney et al., 1984). Ossification begins at the centre of the rudiment and spreads peripherally (Streeter, 1951), such that eventually the developing epiphysis becomes the last remaining cartilaginous area. The first indication of joint formation is the appearance of a dense region of rounded cells which then flatten to form the interzone of the presumptive joint (Mitrovic, 1978; O’Rahilly & Gardner, 1978). Joint morphology is achieved by matrix secretion and cell division in the opposing epiphyses (Craig et al., 1987; Craig, 1988) and finally cavitation occurs to form the joint space. Craig et al., (1990) have shown that the interzone is rich in hyaluronan, but poor in collagen and so could be an area of weakness where cavitation may occur to separate the articular surfaces. In mammals the formation of a secondary centre of ossification within the epiphysis serves to separate the growth plate and articular cartilages (Haines, 1937), which continue to grow at different rates (Hinchliffe & Johnson, 1983). Mankin (1962), Rigal (1962) and Pribylova & Hert (1971) have all described two concentric areas of mitotic activity in the articular cartilage of immature rabbits, one superficially, the second near the
subchondral bone which allow for expansion of the epiphysis and subchondral bone. Proliferation in articular cartilage declines with age such that in mature normal cartilage mitotic figures are not apparent (Mankin, 1963, 1964).

The structure of articular cartilage.

Articular cartilage comprises a small number of cells, the chondrocytes, embedded in a large volume of extra-cellular matrix. The chondrocytes typically occupy only 10% of the total volume of the tissue (Hamerman & Schubert, 1962; Stockwell, 1971a, b; Stockwell & Meachim, 1979). The physiological and mechanical properties of cartilage are largely dependent on the structure of the matrix, which, in turn, is produced and maintained by the chondrocytes. The three major components of cartilage matrix are collagen, which accounts for approximately 50-65% of the dry weight of the tissue (Anderson et al., 1964; Bayliss & Venn, 1980), proteoglycan and hyaluronan, which, although it accounts for less than 1% of the dry weight, is fundamentally important in matrix organisation (Mason, 1981). In cartilage, collagen forms an insoluble fibrillar meshwork which helps to maintain shape and is highly resistant to tension but, in isolation, cannot withstand compressive stresses without collapse (Scott, 1988; Broom, 1988). A proteoglycan, as defined by Hascall and Kimura (1982), is “any macromolecule that has a core protein containing at least one covalently bound glycosaminoglycan chain”, a glycosaminoglycan being a heteropolymer consisting of repeating disaccharide sequences containing hexosamine and hexuronic acid or hexose. The majority of cartilage proteoglycans are capable of interacting specifically with hyaluronan to form high molecular weight aggregates (Hascall & Muir, 1972). The interaction is further stabilised by the presence, in cartilage, of a low molecular weight glycoproteinaceous link protein (Hascall & Muir, 1972). Proteoglycans are polyanionic due to the presence of large numbers of sulphated glycosaminoglycan side chains and so exist in a hydrated form in cartilage and are largely responsible for the swelling pressure of articular cartilage (Maroudas & Venn, 1977). In isolation proteoglycan aggregates are extremely weak in shear (Simon et al., 1989), but when trapped in a compressed state within the collagenous network found in cartilage, the resultant tissue is capable of withstanding high levels of both shear and compressive forces (Meachim & Stockwell, 1979).

Collagen. Collagens are structural molecules which rely, largely, for their function on the typical collagenous triple helix. So far 13 different collagens have been identified in tissues, of which five are known to be present in bovine articular cartilage, namely collagens II, V, VI, IX and XI (Eyre et al., 1987b). The primary structure of all collagens contain long lengths of repetitive amino acid triplets of the form Glycine-X-Y, where X and Y may be any amino acid but are often proline or hydroxyproline (Ramachandran & Kartha, 1955). This primary structure is an absolute requirement for the formation of a right-handed helical structure between three polypeptide α chains as is seen in all collagens (Fietzek & Kuhn, 1976; Miller, 1976; Prockop, 1977). Helices may be formed from three identical α
chains, for example collagen II or from different gene products, as in the case of collagen IX. Interactions between the helical domains of two or more molecules are common and lead to the formation of oligomers or high molecular weight fibrils as found in cartilage. The structure of the different collagen types have been reviewed in detail by the following authors; Miller (1978), Mayne & Von der Mark (1983), Mayne & Irwin (1986), Mayne & Burgeson (1987), Burgeson (1988), Mayne (1989) and a brief description only will be given here.

Collagen II accounts for 90-95% of the total collagen in bovine articular cartilage (Eyre et al., 1987b), and is the major component of the fibrillar collagenous meshwork found in cartilage. It is synthesised as a procollagen possessing both N and C terminal globular extensions which are subsequently cleaved before the molecule is incorporated into fibrils (Mayne & Von der Mark, 1983; Mayne & Burgeson, 1987; Kuhn, 1987). Molecules of collagen II are capable of aggregating in a "quarter stagger" array, with approximately 75% of each molecule in contact with its neighbours in front and behind. This arrangement is determined by the amino acid sequence of the α chains, such that hydrophobic and charge attractions are maximised (Hulmes et al., 1973; Cunningham et al., 1976). Collagen II is the major component of the long, small diameter fibrils typical of cartilage matrix although there is growing evidence that other molecular species are required for the formation and regulation of size of these fibrils (Lee & Piez 1983; Birk & Silver, 1984; Burgeson, 1988).

Collagen V represents approximately 1-2% of the total collagen in bovine articular cartilage (Eyre et al., 1987b). Three genetically distinct α chains have been identified which may be combined in several different permutations (Mayne & Burgeson, 1987). Collagen V is normally found in association with fibrils of type I collagen such as are found in the cornea (Linsenmayer et al., 1985; Birk et al., 1988). The function of this collagen type in cartilage, which normally does not contain type I collagen, is not understood.

Collagen VI accounts for 1-2% of the total collagen of bovine articular cartilage (Eyre et al., 1987b). Type VI collagen possesses a particularly short triple-helical region, 105 nm compared to 300 nm for collagen II, bounded by two globular domains (Furthmayer et al., 1983; Jander et al., 1984; Timpl & Engel, 1987; Chu et al., 1988). Whilst the molecule can exist as a monomer, it is more commonly found as aggregated di or tetramers, bonded by disulphide bridges (Jander et al., 1983; von der Mark et al., 1984). Tetramers can form the basic subunit of microfilaments, which are of much smaller diameter than the normal collagen II fibrils found in cartilage (Keene et al., 1988; Ayad et al., 1989). The tertiary and quaternary structure of collagen VI makes it completely resistant, in its native form, to digestion by bacterial collagenase (Abedin et al., 1982; Jander et al., 1984). In articular cartilage collagen type VI is localised to microfilaments found between the larger interstitial fibrils. It is also found in larger amounts in the fine collagenous capsule which surrounds
chondrocytes (Ayad et al., 1984; Poole et al., 1988a). The function of type VI collagen is, as yet, unknown but it has been suggested that it may help to regulate fibril diameter. It may also provide an adhesive mechanism for cell-substratum interactions and may help protect chondrocytes from compressive loading (Carter, 1982; Bruns et al., 1984; Linsenmayer et al., 1986; Wayner & Carter, 1987; Poole et al., 1988a).

Collagen IX, 1-2% in bovine articular cartilage (Eyre et al., 1987b). It is known to contain three collagenous domains (COL 1-3) and four non-collagenous domains (NC 1-4) (Van der Rest et al., 1985; van der Rest & Mayne, 1987). The molecule is assembled from three genetically distinct chains, one of which, α2(IX), has a single chondroitin sulphate chain covalently attached to a unique serine residue located within the NC 3 region (Vaughan et al., 1985; Bruckner, 1985; Irwin & Mayne, 1986; McCormick et al., 1987; Huber et al., 1988), although this may be lost in mature bovine cartilage (Ayad et al., 1989). Type IX collagen has been shown, using immunolocalisation, to lie periodically along the surface of collagen fibrils and to be bound covalently to collagen II (Muller-Glauser et al., 1986; Eyre et al., 1987a; Vaughan et al., 1988). This has lead to speculation that the molecule may act to restrict the diameter of fibrils (Wotton et al., 1988). Indeed, in bovine cartilage, which contains many thick fibrils, type IX collagen makes up only 1-2% of the total collagen and is preferentially located in the fine fibrils found surrounding chondrocytes (Poole et al., 1988b).

Collagen type XI represents 2-3% of the total collagen in bovine articular cartilage (Eyre et al., 1987b). The 1α and 2α chains are closely related to the equivalent chains of type V collagen, whilst the 3α chain is believed to be an over-glycosylated form of the 1α chain of collagen II (Furuto & Miller, 1983; Eyre et al., 1984; Eyre & Wu, 1987; Morris & Bachinger, 1987; Bernard et al., 1988). Type XI collagen is found in the same fibrils as collagen II although its exact localisation is not known (Vaughan et al., 1988). Theories have been advanced that it may either be found in the centre of fibrils and play a role in the incorporation of type II collagen (Mayne, 1989) or be present at the surface and help to regulate fibril diameter (Smith et al., 1985).

Proteoglycans. The structure and function of cartilage proteoglycans has been extensively reviewed by Carney & Muir, (1988). Two major types of proteoglycan are known to be present in articular cartilage, dermatan sulphate proteoglycan (DSPG) and large aggregating proteoglycans. The yield of DSPG is approximately 5% of the total proteoglycan weight, but when the difference in molecular size of the two species are taken into account they could be present in similar molar amounts (Roughley & White, 1989).

DSPG molecules typically possess a protein core substituted with only one or two glycosaminoglycan side chains, which may be either chondroitin or dermatan sulphate (Coster et al., 1986; Sampaio et al., 1988). DSPG is found in higher concentrations in
juvenile tissue and is known to interact with collagen. This has led to speculation that it is involved in deposition and maintenance of the collagen meshwork of cartilage (Sampaio et al., 1988).

The large aggregating proteoglycans are largely responsible for the compressive properties of articular cartilage due to the high swelling pressure exerted by polyanionic proteoglycan aggregates. Aggregating proteoglycans are composed of a proteinaceous core substituted with large numbers of polyanionic glycosaminoglycan side chains. The two main glycosaminoglycans present in cartilage aggregating proteoglycans are chondroitin sulphate and keratan sulphate. Chondroitin sulphate consists of repeated disaccharide units of glucuronic acid and N-acetyl galactosamine linked β-(1-4) between disaccharide units and β-(1-3) between glucuronic acid and N-acetyl galactosamine (reviewed in Hardingham, 1981; Carney & Muir, 1988). Disaccharides may be sulphated at either the C-4 or C-6 position of galactosamine (Seno et al., 1974, 1975) leading to the terms chondroitin-4-sulphate and chondroitin-6-sulphate. Chondroitin sulphate chains are linked to serine or, occasionally, threonine residues on the proteoglycan core protein via an O-glycosidic bond involving a single xylose sugar (Muir, 1958; Gregory et al., 1964; Anderson et al., 1965; Roden & Armand, 1966). The repeating disaccharide units of keratan sulphate consist of galactosamine linked β-(1-4) to N-acetyl glucosamine; the disaccharides being linked β-(1-3) to each other. The keratan sulphate linkage to core protein serine residues involves an O-glycosidic bond to a branched tetrasaccharide linkage region (Bray et al., 1967; Hopwood & Robinson, 1974). In addition the core protein contains O-linked oligosaccharides present throughout its length and N-linked oligosaccharides, present at the hyaluronan-binding region (Thonar & Sweet, 1979; De Luca et al., 1980).

The proteoglycan core protein contains several distinct regions, identified by structure or function. The hyaluronan-binding region (HABR) comprises approximately a quarter of the total protein (Hascall & Heinegard, 1974; Heinegard & Hascall, 1974). This region is highly folded into a globular structure, containing between five and seven disulphide bridges which are essential for binding of core protein to hyaluronan (Hardingham et al., 1976). Next to the HABR is the keratan sulphate-rich region, which by definition bears the majority of the keratan sulphate chains, but only a small proportion of the chondroitin sulphate chains (Heinegard & Axelsson, 1977). This region of the core protein is believed to be rich in glutamic acid, glutamine, proline and serine. The bulk of proteoglycan core protein is referred to as the chondroitin sulphate-rich region, as it contains the majority of the chondroitin sulphate chains and also approximately 30% of the keratan sulphate chains (Heinegard & Axelsson, 1977). The length of the chondroitin sulphate-rich region is highly variable, probably due the susceptibility of this region for proteolytic cleavage, liberating chondroitin sulphate-peptide clusters (Mathews, 1971; Heinegard & Hascall, 1974). In addition to the HABR, there are believed to be two other globular domains within the core protein, termed G2 and G3 (Wiedemann et al., 1984). It has been
tentatively suggested that these regions are responsible for binding cartilage matrix protein in the extracellular matrix (Lord et al., 1984).

**Hyaluronan.** Hyaluronan (HA) is an unbranched glycosaminoglycan consisting of repeating disaccharide units of glucuronic acid linked β-(1,3) to N-acetylglucosamine. The disaccharides are linked to each other by β-(1,4) linkages (Mason, 1981). Whilst the molecular weight is variable in cartilage, it can exceed $1 \times 10^6$. Hyaluronan accounts for less than 1% of the dry weight of bovine articular cartilage (Thonar et al., 1978), but is essential for the formation of high molecular weight HA-PG aggregates. There is a tendency for the HA content of cartilage to increase with age (Bayliss & Ali, 1978; Thonar et al., 1978).

Cartilage proteoglycans aggregate with HA to form large aggregates, which are stabilised by a third component, link protein (Hardingham & Muir, 1972; Hardingham, 1979). Link protein stabilised HA-PG aggregates are primarily responsible for the swelling pressure in articular cartilage, which is vital for cartilage function. The interaction is highly specific and occurs via the proteoglycan hyaluronan binding region (HABR) and requires intact disulphide bridges within the HABR globular domain (Hardingham et al., 1976). Proteoglycan monomers will bind optimally to decasaccharides of HA but not to smaller molecules (Hascall & Heinegard, 1974). Whilst HA-PG binding is strong, it is reversible in the absence of link protein. When link protein is present, however, under physiological conditions the interaction is essentially irreversible (Hardingham, 1979; Tang et al., 1979). Three different link proteins have been identified, all of which can bind to both HA and PG monomers (Heinegard & Hascall, 1974; Keiser, 1975; Baker & Caterson, 1979; Franzen et al., 1981). One molecule of link protein is believed to bind stoichiometrically to one molecule of PG monomer and HA. Whilst PG monomer may bind optimally to an HA decasaccharide, PG-link protein complex requires an HA oligosaccharide containing at least 24 monosaccharides (Hardingham et al., 1983). It has been suggested that PG-link protein complex, when bound to HA, may protect a 50 monosaccharide section of HA from hyaluronidase attack (Faltz et al., 1979).

**Matrix turnover in articular cartilage.**

In normal adult articular cartilage there is a slow, but steady turnover of matrix components so that components lost due to degradation are replaced by newly synthesised molecules (Handley et al., 1986). This is vital, *in vivo*, to maintain the structural and functional integrity of the cartilage. The turnover rate of collagen in mature cartilage is slow, as indicated by very low levels of collagen synthesis and degradation in adult tissue when compared with embryonic (Repo & Mitchell, 1971; Juva et al., 1966). It appears, therefore, that the collagenous component of cartilage is laid down during development and normally changes little thereafter (Muir, 1979). Proteoglycans are turned over at a much faster rate, with half-lives ranging from 45 days in the young rabbit to 800 days in the human.
femoral head (Maroudas & Evans, 1974; Maroudas, 1975; Fassbender, 1986; Morales & Hascall, 1989). This means that maturation and age-related proteoglycan changes are possible and, indeed, do occur in articular cartilage. In general, during normal aging there is an increase in the content of keratan sulphate and protein and a decrease in the chondroitin sulphate content and average hydrodynamic size of dissociated proteoglycans (McDevitt & Muir, 1971; Inerot et al., 1978). An increase in the variation in size of proteoglycan monomers has also been observed (Buckwalter & Rosenberg, 1983) but no significant difference in the ability to aggregate with hyaluronate has been noted (Bayliss & Ali, 1978; Roughley & White, 1980). Once sexual and skeletal maturity is reached changes occur at a much slower rate with the quantity and type of glycosaminoglycan present changing very little (Elliott & Gardner, 1979). Changes which occur during development and aging are believed to be caused primarily by continuous, gradual proteolytic cleavage of matrix proteoglycan core protein and link protein (Sweet et al., 1979; Roughley & White, 1983; Mort et al., 1983; Mort et al., 1985). An alteration in the synthesis of both the protein and carbohydrate components of proteoglycans may also play a role during aging (Hjertquist & Lemperg, 1972; Hjertquist & Wasteson, 1972; Honda et al., 1979; Theocaris et al., 1985). Matrix turnover appears to be controlled by a combination of cell shape and microenvironment, as indicated by altered metabolism and phenotype seen when chondrocytes are removed from their matrix and cultured in isolation (Benya & Brown, 1986; Watt & Dudhia, 1988). Mechanical and humoral factors are also believed to be involved in the maintenance of matrix integrity (Slowman & Brandt, 1986; Morales & Hascall, 1989).

Abnormal and diseased articular cartilage.

Whilst alterations in the composition of matrix molecules have been reported there is little evidence to suggest that these changes adversely affect the functional properties of normal healthy articular cartilage, as indicated by the presence of humans and animals of advanced age with functionally intact joints (Freeman & Meachim, 1979; Roughley & Mort, 1986; Carney & Muir, 1988). Certain conditions do exist, however, where the balance between synthesis and degradation is upset and in many cases this leads to disfunction. In early acromegaly increased pituitary secretions lead to an increase in matrix production and the formation of thickened cartilage (Sokoloff, 1969). Degenerative changes where degradation exceeds synthesis are much more common, with osteoarthritis being the most widespread and clinically important. Osteoarthritis is a disease common in older people and is characterised by a net loss of matrix proteoglycans and a disruption in articular cartilage architecture. Eventually lesions become apparent and the progressive nature of the disease leads to exposure of the underlying subchondral bone (Mankin, 1974; Freeman & Meachim, 1979; Sokoloff, 1983). Whilst much of the work performed on osteoarthritis has utilised animal models of the disease, similarities to the changes observed in the human disease have been reported. An increase in water content and swelling of cartilage are features of the disease and may be due to weakening of the collagen network, although no
change in overall collagen content have been noted (Mankin & Lippiello, 1970; Maroudas, 1976a; McDevitt & Muir, 1976; Orford et al., 1983; Broom, 1984). Changes in the structure of proteoglycans have been shown, including an increase in the proportion of chondroitin sulphate compared to keratan sulphate which is believed to be due to an increase in both length and number of chondroitin sulphate side chains (McDevitt et al., 1975; McDevitt & Muir, 1976; McDevitt et al., 1977; Vasan, 1980). A decrease in protein and hyaluronan content has also been reported (Eguchi et al., 1974; Muir, 1986). The chondrocytes from osteoarthritic cartilage show metabolic changes which are believed to be an attempt by the cells to repair the damaged tissue. The lack of vascularisation in articular cartilage, which normally plays a central role in reparative processes, and the slow metabolic turnover rate of chondrocytes means that repair is usually unsuccessful (Mankin, 1982). Many investigators have reported an increase in synthetic rates of proteoglycans (Mankin & Lippiello, 1970; Mankin et al., 1971; McDevitt et al., 1981; Sandy et al., 1984), collagen (Eyre et al., 1980; Floeman et al., 1980) and hyaluronan (Ryu et al., 1984) in both human and animal model osteoarthritic cartilage but this is insufficient to counter the levels of degradation present.

Another notable feature of osteoarthritis is the appearance of chondrocyte clusters within the cartilage. Several groups have demonstrated an increase in DNA synthesis, as indicated by tritiated thymidine labelling, and have proposed that cell division is involved in cluster formation and is another indication of an attempted repair process (Telhag, 1972; Hulth et al., 1972; Rothwell & Bentley, 1973; Hirotani & Ito, 1975; Havdrup & Telhag, 1978). Whilst some workers have reported an increase in total cellularity in arthritic cartilage (Mankin & Lippiello, 1970; Mankin et al., 1971), others have shown no change (Pelletier et al., 1985) or even a reduction in cell numbers (Vignon et al., 1974). This inconsistency, coupled with the acellularity of the tissue normally found surrounding clusters has cast doubt on the above theory.

Although the metabolic processes in normal and arthritic chondrocytes have been well documented, the control of such mechanisms are poorly understood. Factors which may affect chondrocyte metabolism include cell shape, cell-matrix interactions, loading, growth factors, matrix hindrance and receptor expression (Sokoloff, 1985; Morales & Hascall, 1989). An understanding of the factors controlling chondrocyte metabolism and its alteration in diseased states is, therefore, vital for the future advancement of arthritis research and the development of new treatments.

**Project aims.**

The aim of this thesis is to study the morphological, metabolic and biochemical changes which occur in adult articular cartilage when it is subjected to an insult sufficient to upset the synthetic-degradative equilibrium found in normal cartilage. This has been achieved by treating cartilage explants, *in vitro*, with enzymes specific to matrix compo-
tems which cause depletion of matrix, alteration in cartilage architecture, or both. After enzyme treatment, the cartilage was cultured for a further two weeks, allowing the investigators to gain an insight into any changes and possible repair processes which may occur within the tissue. The two enzymes under investigation are highly purified collagenase and hyaluronidase, both of bacterial origin, chosen due to the central role played by collagen and hyaluronan in matrix structure. Studies of this kind have been attempted previously but using enzymes of lower purity and specificity (Bentley, 1971; Harris et al., 1972; Hardingham et al., 1972; Bartholomew et al., 1985; Verbruggen et al., 1985a,b; Moriizumi, Yamashita & Okada, 1986). Results variously showed an increase in matrix synthesis (Hardingham, Fitton-Jackson & Muir, 1972; Verbruggen et al., 1985a,b), a reduction in matrix synthesis (Bartholomew et al., 1985), reduction in the stability of proteoglycan (Harris et al., 1972; Verbruggen et al., 1985a,b) and the onset of cell division (Bentley, 1971; Havdrup, 1979; Verbruggen et al., 1985a; Moriizumi et al., 1986). It is hoped that the present work may be able to resolve some of the anomalies present in previous studies. The culture system chosen utilises full-depth cartilage explants from the bovine metacarpalphalangeal joint. Explant cultures, unlike cultures of isolated chondrocytes, have the advantage of retaining cartilage architecture but are not complicated by the presence of other joint tissues, such as occurs when in vivo systems are used. Bovine cartilage was chosen because it is easy to obtain young adult tissue in large quantities from the local abattoir and also because it has been well characterised by other workers (Hascall et al., 1983; Handley et al., 1986; Luyten et al., 1988).

This section is intended as a general introduction and as a background to the work undertaken. Each subsequent chapter contains its own introduction outlining points relevant to that chapter in more detail.
CHAPTER 1.

THE LOSS OF MATRIX COMPONENTS FROM ENZYME TREATED ARTICULAR CARTILAGE.
Introduction

As a covering to the articular ends of bones, cartilage provides both protection from stress damage and a highly lubricated, almost frictionless surface which is required for articulation of the bones. Articular cartilage function relies largely on the structure of the matrix and, consequently, on the structure and interactions of the molecular components which comprise the matrix. Whilst water accounts for about 70% of the weight of normal cartilage, the resulting 30% (the dry weight) is broken down as follows; 50-65% collagen, 35% proteoglycan and less than 1% hyaluronan. Other components, such as non-collagenous protein and lipid are also present in variable amounts (Anderson et al., 1964; Stockwell, 1979). Collagen forms an insoluble fibrillar meshwork in which proteoglycan-hyaluronan aggregates are trapped, the resulting tissue being able to withstand high levels of both compressive and shear forces. The matrix is maintained by a continuous process of synthesis and loss of matrix components. In certain diseased states matrix homeostasis is upset, leading to net matrix depletion and, eventually, to disfunction. The aim of the present study is to investigate the effect of upsetting the balance between matrix synthesis and loss. This has been achieved, in vitro, by incubating explants of bovine articular cartilage with either hyaluronidase or collagenase.

Hyaluronidase (EC 4.2.2.1), Type IX, isolated and purified from Streptomyces hyalurolyticus has been used throughout. Ohya & Kaneko (1970) showed that this enzyme exhibits an absolute specificity for hyaluronan and, unlike testicular hyaluronidase, will not cleave chondroitin or keratan sulphate (Ohya, 1971; Yamada, 1973; Yamada & Hirano, 1973; Derby & Pintar, 1978). The enzyme carries out an elimination reaction yielding hyaluronan oligosaccharide residues with 4, 5-unsaturated glucuronosyl residues at the non-reducing end (Meyer, 1971; Shimada & Matsumura, 1980). Shimada & Matsumura, (1980) have also shown that octasaccharides were the minimum sized substrates to be cleaved and that hexa- and tetrascaccharides were the end product of digestion.

The collagenase used is a highly purified version of the enzyme first isolated from Clostridium histolyticum by Mandl and coworkers in 1953 and subsequently numbered EC 3.4.24.3. The enzyme preparation contains two similar but distinct activities, termed collagenases A and 2 (Mandl et al., 1964; Yoshida & Noda, 1965; Mitchell, 1968; Schaub & Strauch, 1968; Harper & Kang, 1970). Clostridium collagenases are endopeptidases, cleaving collagen preferentially at the Y-Gly bond in the sequence Pro-Y-Gly-Pro (Mandl, 1961; Harper & Kang, 1970; Seifter & Harper, 1971; Lwebuga-Mukasa et al., 1976; Mookhtiar et al., 1985). In contrast, mammalian collagenases, which have been implicated in joint diseases, cleave collagen at a specific site yielding fragments representing $\frac{1}{4}$ and $\frac{3}{4}$ of the molecule and so comparisons should be made with extreme caution (Gross et al., 1974; Woolley et al., 1975).
Whilst the degradative processes of both *Streptomyces* hyaluronidase and *Clostridium* collagenase on purified substrates are well known, the effects on complex tissue are less well understood. Yamada, (1981) reported that sulphated glycosaminoglycans as well as hyaluronan were lost from cartilaginous tissue sections when treated with hyaluronidase. Hyaluronidase has been shown to affect tissue organisation, neurulation and cell cycle time in embryos, (Schoenwolf & Fisher, 1983; Morriss-Kay et al., 1986; Tucker & Morris-Kay, 1989). Capuo & Raisz, (1980) reported an increased loss of proteoglycan from foetal rat cartilage cultured with the enzyme. The effect of *Streptomyces* hyaluronidase on the metabolism of adult articular cartilage, however, is unclear. Whilst *Clostridium* collagenase has been used extensively in the preparation of chondrocyte cultures, the effect at concentrations insufficient to cause total matrix destruction is less well characterised. Harris et al., (1977) demonstrated an increase in the loss of both hydroxyproline and hexosamine from collagenase treated cartilage, with an accompanying loss of both thickness and stiffness. Broom (1988) observed changes in collagenase-treated cartilage consistent with a breakdown in the collagen fibril interlinking system prior to total destruction. Other similar studies have employed enzymes of lower purity or specificity, such as testicular hyaluronidase (Jackson, 1970; Hardingham et al., 1972; Verbruggen et al., 1985a, b), trypsin (Bartholomew et al., 1984) or papain (Bentley, 1971; Morizumi et al., 1986).

It is of importance to establish the extent of matrix depletion caused by the enzymes, so that any observed metabolic changes can be correlated with cartilage matrix component loss. The aim of this first chapter is, therefore, to measure the loss of matrix components into the medium of bovine articular cartilage explants incubated with either *Streptomyces* hyaluronidase or *Clostridium* collagenase.

**Materials and Methods**

**Culture medium**

Except where stated, the medium used in all culture experiments was as follows; Dulbecco’s modification of Eagle’s minimal essential medium containing 3.7 mg.ml\(^{-1}\) sodium bicarbonate and 4.5 mg.ml\(^{-1}\) D-glucose (Flow Labs., Irvine, Scotland) supplemented with 2mM L-glutamine, 20mM HEPES buffer, antibiotic/mycotic solution (100 unit.ml\(^{-1}\) penicillin, 100 mg.ml\(^{-1}\) streptomycin and 0.25 mg.ml\(^{-1}\) fungizone) and 100 unit.ml\(^{-1}\) nystatin (all Gibco Ltd, Paisley, Scotland). The term DMEM has been used subsequently to describe the medium above.
Cartilage explant cultures

The front feet from 1-2 year-old steers were obtained from the local abattoir and stored at 4°C prior to dissection (24 hr. maximum). The feet were washed and sterilised by immersion in 70% alcohol for 5 min. after which the metacarpal-palangeal joint was opened under aseptic conditions (see Fig. 1.1). Full depth pieces of articular cartilage were dissected out and washed briefly in phosphate-buffered saline (PBS). The explants were then transferred to 35mm Falcon tissue culture dishes, (2 explants per dish) containing 2ml DMEM with 20% (v/v) foetal calf serum (FCS) and twice normal concentration of antibiotic/mycotic as described previously. The cultures were incubated at 37°C in 5% CO₂ in air in a Flow tissue culture incubator for 3 days, to allow equilibration to culture conditions and to check for infection, prior to the start of experiments. Subsequently, the medium was replaced with fresh DMEM + 20% FCS every two days except where stated otherwise.

Enzymic depletion of cartilage

Explant cultures were set up as previously described and allowed to equilibrate to culture conditions for 3 days prior to enzyme treatment. The medium was then removed and replaced with DMEM + 20% FCS containing the enzyme to be investigated and the cultures incubated for 24 hours at 37°C. At the end of this period the enzyme-containing medium was replaced with fresh medium without the enzyme. During subsequent culture the medium was replaced every two days. Control cultures were incubated for 24 hours in DMEM + 20% FCS without enzymes.

The enzymes used were:

- Hyaluronidase (EC 3.2.1.35), Type IX from *Streptomyces hyalurolyticus* (Sigma, Poole, England). Enzyme used at a concentration of 10 unit.ml⁻¹.
- Collagenase (EC 3.4.24.3), chromatographically purified from *Clostridium histolyticum*, (Worthington Diagnostic Systems, Reading, England). Enzyme used at concentrations of either, 20 unit.ml⁻¹, or 100 unit.ml⁻¹.

Time 0, for all experiments was taken as the time when the enzymes were added.

Dose response of cartilage explants to hyaluronidase or collagenase

Bovine cartilage explants were removed as described above and cultured in DMEM + 20% FCS for 3 days. At the end of this period, fresh medium was added containing either hyaluronidase or collagenase and the cultures incubated for a further 24 hours. The enzyme concentrations used were:

- Hyaluronidase: 5, 10, 20 and 50 unit.ml⁻¹.
- Collagenase: 10, 20, 50 and 100 unit.ml⁻¹.

At the end of this period the medium was removed and stored prior to analysis. The tissue was removed from culture and the proteoglycans extracted under associative
conditions, in 1 ml PBS and under dissociative conditions, in 1 ml 4M guanidine hydrochloride (Gu.HCl) made up in 20mM sodium acetate buffer (Sajdera & Hascall, 1969; Bayliss et al., 1983). Both extractions were performed at 4°C for 24 hr. The non-extractable sulphated glycosaminoglycan (GAG) fraction was solubilised by digestion in a solution containing 1μl papain suspension (type III, Sigma, Poole, England) in 1 ml PBS supplemented with 5 mM cysteine hydrochloride and 5 mM ethylenediaminetetraacetic acid (EDTA) at pH 5.7 for 24 hours at 60°C (Scott, 1960; Arnon, 1970; McDevitt et al., 1974). The total amount of GAG was determined in both papain digests and medium by a modification of the method reported by Farndale et al., (1982) as described below. Enzymic depletion was expressed as percentage loss of total sulphated GAG into the medium. GAG content was determined for medium, PBS and guanidine HCl extracts and papain digest and used to determine the proportion of total sulphated GAG in each fraction.

Loss of matrix components from explants into the medium

Explants were set up as described above and digested with either hyaluronidase (10 unit.ml⁻¹) or collagenase (20 or 100 unit.ml⁻¹). Medium samples were removed and stored when the cultures were fed on days 1, 3, 5, 7 and 9. On day 9 the explants were removed from culture and digested in 1ml papain solution at 60°C for 16 hours. The total amount of sulphated glycosaminoglycan, collagen and hyaluronan in medium samples and papain digests was determined and expressed as a percentage loss into medium as follows-

\[
\]

Determination of glycosaminoglycan concentration in tissue and medium:

Glycosaminoglycan (GAG) concentration in papain digests of cartilage and culture medium was determined by a modification of the 1, 9-dimethylmethylene blue (DMB) dye binding assay described by Farndale, Sayers and Barrett (1982). The assay relies on a metachromatic shift in absorption maximum from 600 nm to 535 nm when the DMB dye is complexed with sulphated GAG (Taylor & Jeffree, 1969, Humbel & Etringer, 1974). Forty microlitre samples of either papain digest or culture medium were aliquoted into microtitre wells to which was added 250 μl DMB (Serva Feinbiochemica, Heidelberg, Germany) and the absorbance at 600 nm, A₆₀₀read immediately using a Bio-Rad model 2550 EIA plate reader. The A₆₀₀ values were compared with a standard curve prepared using
shark chondroitin-4-sulphate (Sigma, Poole, England).

**Determination of hydroxyproline concentration in tissue and medium**

Hydroxyproline is an imino acid found almost exclusively in collagen, (Berg & Prockop, 1976). A large proportion, up to 17%, of the amino acid residues found in normal collagen are hydroxyproline (Scott, 1988) and so most quantitative collagen assays involve the oxidation of hydroxyproline to pyrrole derivatives which are further reacted to give a coloured product which can be detected spectrophotometrically. The method described is similar to that reported by Woessner (1976). The method does not give absolute collagen concentrations but because the experimental results are expressed as percentages this is unimportant.

The tissue and medium samples were hydrolysed by incubation with 0.5 ml of 12 N HCl and 0.5 ml water at 130°C for 3 hr. After cooling the reaction mixtures were brought to pH 6 by adding saturated LiOH and made up to 5ml with water. The mixtures were then oxidised by the addition of 1ml propan-2-ol and 200 µl chloramine-T solution for 20 min after which 0.5 ml perchloric acid reagent was added. Colour was developed by incubation for 20 min. at 60°C with 1 ml freshly diluted p-dimethylaminobenzaldehyde made up in propan-2-ol. After cooling the absorbance at 562 nm was read using a Philips PU 8720 spectrophotometer and compared to hydroxyproline standards prepared in the same way.

**Determination of hyaluronan concentration in tissue and medium:**

The assay used to determine the concentration of hyaluronan is as described by Fosang et al., (1990). The assay involves competition between hyaluronan absorbed onto microtitre plates and hyaluronan, free in solution, for binding to biotinylated cartilage proteoglycan binding region (biotinylated-G1 domain). Plastic microtitre plates (Immunolon, Dynatech) were coated overnight with 200 µl of a 25 µg ml solution of hyaluronan (human umbilical cord, BDH, Poole, England) in 20mM sodium carbonate coating buffer (pH 9.6). The coating solution was removed and the plates washed in PBS + 0.05% (w/v) Tween 20 (PBS / Tween), and then blocked for 90 min at 37°C in 1% (w/v) bovine serum albumin (BSA) in PBS / Tween.

Samples (100µl) or hyaluronan standards (10ng-20µg.ml⁻¹), in PBS / Tween were added to the wells, followed by 100µl of 1µg.ml⁻¹ biotinylated-G1 diluted in PBS / Tween. Blank wells contained 200µl PBS / Tween whilst maximum binding was determined from wells containing 100µl PBS / Tween and 100µl biotinylated G1. The plates were stood for 48 hr at 4°C and then washed 4 times in PBS / Tween.

After washing, 200µl of 1µg.ml⁻¹ streptavidin -horseradish peroxidase conjugate diluted in PBS and containing 1% (w/v) BSA was added to all wells and incubated at 37°C for 30 min. The plates were washed 4 times in PBS / Tween and then 200µl of the peroxidase substrate, 2, 2' Azino-di-[3 ethylbenzthiazoline sulphonate] (ABTS, Kirkegaard & Perry) Labs, Maryland, USA) was added to the plates and incubated at room temperature. Colour
was added to the plates and incubated at room temperature. Colour development was monitored at 405 nm and the plates were read when the optical density of the wells containing biotinylated G1 only (maximum binding) had reached 1.5. The plates were read using a Bio-Rad model 2550 EIA plate reader set to 405 nm. Hyaluronan concentrations in the samples were determined from the standard curve.

**Results.**

**Dose response of cartilage to hyaluronidase and collagenase:**

In order to select enzyme doses to be investigated further in subsequent experiments, it was first necessary to quantify the effect of different concentrations of the two enzymes on the loss of matrix from treated cartilage explants. The loss of sulphated glycosaminoglycan (GAG) from the tissue was used as a guide as neither enzyme actively attacks the molecule, but both were found to cause passive loss. In order to gain a further insight into the mode of component loss, the total tissue glycosaminoglycan was compartmentalised into medium, PBS extract, guanidine HCl extract and papain residue. The percentage of total tissue glycosaminoglycan in each fraction for control, hyaluronidase-treated and collagenase-treated has been expressed in Fig. 1.2 (page 27).

Statistical analyses of the data presented were performed as follows. First, analysis of variance tests were used to determine whether any significant differences exist between treatment groups. Analysis of variance test results are presented in Fig. 1.3 (page 28) for hyaluronidase-treated explants and in Fig. 1.4 (page 29) for collagenase-treated explants. Hyaluronidase was found to increase, significantly, the proportion of GAG present in the medium and PBS fractions at the expense of GAG present in the guanidine HCl extract. The proportion of GAG present as inextractable residue, solubilisible only with papain, was found to be unchanged in hyaluronidase-treated explants as compared to controls. Collagenase also increased the proportion of GAG in the medium and PBS extracts, but at the expense of both the guanidine HCl extract and non-extractible papain residue.

Whilst a positive result suggests that enzyme treatment does affect the parameter being measured, it gives no indication of statistical differences between treatment groups. Multiple Student's t-test analysis is not appropriate in this instance (Janssen, 1986) and so Newman-Keuls tests, a variation of the t-test statistic specifically designed for multiple comparisons, have been employed (Glantz, 1987). Tabulated results are presented in Fig. 1.5 (page 30) for hyaluronidase-treated explants and Fig. 1.6 (page 31) for collagenase-treated explants. Hyaluronidase appears to reach maximal response at concentrations above 5 unit.ml\(^{-1}\) whilst collagenase exhibits a dose related response up to 100 unit.ml\(^{-1}\). In view of this finding it has been decided to use only a single concentration of hyaluronidase (10 unit.ml\(^{-1}\)) in further experiments whilst two concentrations of collagenase have been employed (20 & 100 unit.ml\(^{-1}\)).
Loss of sulphated glycosaminoglycan from explants into the medium

In order to determine the loss of GAG into the medium from explants during culture, medium samples, removed on day 1, 3, 5, 7 and 9 and tissue digests were assayed for GAG. Results have been expressed as a cumulative % loss into the medium and have been presented graphically in Fig. 1.7 (pages 32-35). Unpaired Student's t-test analysis has been used to compare treated values with controls (explants incubated in DMEM + 20% FCS). The half-life (t_{1/2}) of proteoglycans in control cultures was approximately 6.5 days and approximately 5 days in serum-free cultures.

Loss of hydroxyproline from explants into the medium

In order to determine the loss of collagen and its breakdown products into the medium from explants during culture, medium samples removed on day 1, 3, 5, 7 and 9 were assayed for hydroxyproline. Medium alone, was found to contain small levels of hydroxyproline, whether it contained serum or not. The increased levels in serum-containing medium may be due to hydroxyproline-containing collagen breakdown products or to other molecules containing the imino acid, such as C1q (Reid, 1982). Medium samples, which had not been cultured with tissue were, therefore, employed as controls, assayed for the presence of hydroxyproline and the mean values subtracted from medium sample concentrations prior to calculation of total percentage loss of hydroxyproline from the tissue. The following table (Table 1.1) represent the mean concentration of hydroxyproline for DMEM + 20% FCS and DMEM alone.

<table>
<thead>
<tr>
<th>Hydroxyproline concentration / μg.ml(^{-1})</th>
<th>Mean.</th>
<th>Standard deviation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM + 20% FCS.</td>
<td>21.5</td>
<td>8.3</td>
</tr>
<tr>
<td>DMEM.</td>
<td>3.7</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Table 1.1. The hydroxyproline content of medium, with and without serum.

Results have been expressed as a cumulative % loss of hydroxyproline into the medium and have been presented graphically in Fig. 1.8 (pages 36-39). Unpaired Student's t-test analysis has been used to compare treated values with controls (explants incubated in DMEM + 20% FCS).

Loss of Hyaluronan from explants into the medium:

The assay used to measure hyaluronan relies on the binding of biotinylated PG binding region to hyluronan. It has been reported that PG binding region requires an oligosaccharide of hyaluronan of at least 10 monosaccharides (Christner et al., 1977, 1979). Since hyaluronidase maximally digests hyaluronan to hexasaccharides (Shimada & Matsumura, 1980), it is possible that short hyaluronan oligosaccharides may be present in...
hyaluronidase-treated cultures which are too small to be detected by the assay. In order to investigate this possibility total hyaluronan in the system (tissue and medium) has been calculated per mg OH-proline. Results presented in Table 1.2.

<table>
<thead>
<tr>
<th>g H/ mg OH-Pro.</th>
<th>Control</th>
<th>Serum-Free</th>
<th>10 unit.ml⁻¹ Hyaluronidase</th>
<th>20 unit.ml⁻¹ Collagenase</th>
<th>100 unit.ml⁻¹ Collagenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean. (Stand. Dev.)</td>
<td>49.4 (12.1)</td>
<td>46.8 (7.6)</td>
<td>33.0 (5.4)</td>
<td>52.8 (11.6)</td>
<td>51.0 (16.3)</td>
</tr>
<tr>
<td>t-test result</td>
<td>p ≥ 0.05</td>
<td>0.05 &gt; p ≥ 0.01</td>
<td>p ≥ 0.05</td>
<td>p ≥ 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2. Table indicating total measurable hyaluronan, presented as g. hyaluronan per mg. hydroxyproline in explants cultured as indicated.

In order to determine the loss of Hyaluronan into the medium from explants during culture, medium samples, removed on day 1, 3, 5, 7 and 9 and tissue digests were assayed for hyaluronan. Results have been expressed as a cumulative % loss into the medium and have been presented graphically in Fig. 1.9 (pages 40-43). Unpaired Student's t-test analysis has been used to compare treated values with controls (explants incubated in DMEM + 20% FCS).
Fig. 1.1. The metacarpalphalangeal joint from an 18 month-old steer, opened ready for dissection of cartilage explants. Cartilage was removed from the surfaces marked with arrows.
Fig. 1.2a. The proportion of total sulphated glycosaminoglycans present in medium, PBS and guanidine hydrochloride extracts and papain digest from explants incubated with various doses of hyaluronidase for 24 hours. Each column represents the mean value of five replicates. Error bars indicate the mean plus the standard deviation of the mean.

- Control.
- 5 unit.ml⁻¹ Hyaluronidase.
- 10 unit.ml⁻¹ Hyaluronidase.
- 20 unit.ml⁻¹ Hyaluronidase.
- 50 unit.ml⁻¹ Hyaluronidase.

Fig. 1.2b. The proportion of total sulphated glycosaminoglycans present in medium, PBS and guanidine hydrochloride extracts and papain digest from explants incubated with various doses of collagenase for 24 hours. Each column represents the mean value of five replicates. Error bars indicate the mean plus the standard deviation of the mean.

- Control.
- 10 unit.ml⁻¹ Collagenase.
- 20 unit.ml⁻¹ Collagenase.
- 50 unit.ml⁻¹ Collagenase.
- 100 unit.ml⁻¹ Collagenase.
<table>
<thead>
<tr>
<th>Source</th>
<th>D of F:</th>
<th>Sum Squares:</th>
<th>Mean Square:</th>
<th>F-test:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>4</td>
<td>2527.013</td>
<td>631.753</td>
<td>29.995</td>
</tr>
<tr>
<td>Within Groups</td>
<td>15</td>
<td>315.933</td>
<td>21.062</td>
<td>p ≤ 0.0001</td>
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<tr>
<td>Total</td>
<td>19</td>
<td>2842.946</td>
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**Fig. 1.3a. Medium.**

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<td>Between Groups</td>
<td>4</td>
<td>27.233</td>
<td>6.808</td>
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<td>Within Groups</td>
<td>15</td>
<td>12.053</td>
<td>0.804</td>
<td>0.0001 &lt; p ≤ 0.005</td>
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<tr>
<td>Total</td>
<td>19</td>
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**Fig. 1.3b. PBS.**

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<td>Between Groups</td>
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<td>2562.717</td>
<td>640.679</td>
<td>32.858</td>
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<tr>
<td>Within Groups</td>
<td>15</td>
<td>292.473</td>
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<tr>
<td>Total</td>
<td>19</td>
<td>2855.19</td>
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**Fig. 1.3c. Guanidine HCl.**

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<td>Between Groups</td>
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<td>58.766</td>
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<tr>
<td>Within Groups</td>
<td>15</td>
<td>509.962</td>
<td>33.997</td>
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**Fig. 1.3d. Papain.**

**Fig. 1.3.** Tabulated results of one way, five group, analysis of variance test used to compare the percentage of total sulphated glycosaminoglycans present in the medium (Fig.1.3a), PBS extract (Fig. 1.3b.), 4M guanidine hydrochloride extract (Fig. 1.3c.) and papain digest (Fig. 1.3d.) from control explants and explants treated with various concentrations of hyaluronidase (5, 10, 20 and 50 unit.ml⁻¹). A p-value of less than 0.05 has been to taken to indicate a significant difference between the treatment groups. Newman-Keuls multiple comparison test have been used to determine differences between treatment groups (results tabulated in Fig. 1.5).
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<td>46.569</td>
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**Fig. 1.4a.** Medium.

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<td>Total</td>
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**Fig. 1.4b.** PBS.

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</tr>
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<td>Within Groups</td>
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<td>0.005 &lt; p ≤ 0.01</td>
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**Fig. 1.4c.** Guanidine HCl.

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**Fig. 1.4d.** Papain.

**Fig. 1.4.** Tabulated results of one way, five group, analysis of variance test used to compare the percentage of total sulphated glycosaminoglycans present in the medium (Fig. 1.4a), PBS extract (Fig. 1.4b), 4M guanidine hydrochloride extract (Fig. 1.4c), and papain digest (Fig. 1.4d) from control explants and explants treated with various concentrations of collagenase (10, 20, 50 and 100 unit.ml⁻¹). A p-value of less than 0.05 has been taken to indicate a significant difference between the treatment groups. Newman-Keuls multiple comparison test have been used to determine differences between treatment groups (results tabulated in Fig. 1.6).
Fig. 1.5. Tabulated results of Newman-Keuls multiple comparison test on % total sulphated glycosaminoglycan present in the medium (Fig. 1.5a.), PBS extract (Fig. 1.5b.), 4M guanidine hydrochloride extract (Fig. 1.5c.) and papain digest (Fig. 1.5d.) from control explants and explants cultured with various concentrations of hyaluronidase (5, 10, 20 and 50 unit.ml$^{-1}$). In all cases treatment means have been ranked in ascending order. NS indicates that the difference between the means of the treatments were not significant at the 5% level whilst * indicates that the difference was significant at the 5% level.
Fig. 1.6. Tabulated results of Newman-Keuls multiple comparison test on % total sulphated glycosaminoglycan present in the medium (Fig. 1.6a.), PBS extract (Fig. 1.6b.), 4M guanidine hydrochloride extract (Fig. 1.6c.) and papain digest (Fig. 1.6d.) from control explants and explants cultured with various concentrations of collagenase (10, 20, 50 and 100 unit.ml⁻¹). In all cases treatment means have been ranked in ascending order. NS indicates that the difference between the means of the treatments were not significant at the 5% level whilst * indicates that the difference was significant at the 5% level.
Fig. 1.7a. Graph representing the loss of sulphated glycosaminoglycans into the medium of explants incubated with enzymes for 24 hours (from Day 0-Day 1) and then cultured for a further 8 days. Each point represents the mean value of five replicates.

-□—  Control.
-♦— Cultured without serum.
-■—  10 unit.ml⁻¹ hyaluronidase.
-♦—  20 unit.ml⁻¹ collagenase.
-▲—  100 unit.ml⁻¹ collagenase.

For clarity, each graph has been plotted individually in Fig. 1.7b-f. These figures include error bars and an indication of the difference, significant or otherwise between treated and control points.

Fig. 1.7b. Graph representing the loss of sulphated glycosaminoglycans into the medium of explants cultured for 9 days in DMEM + 20% FCS (Control). Error bars indicate the mean ± standard deviation of the mean.

Fig. 1.7c. Graph representing the loss of sulphated glycosaminoglycans into the medium of explants cultured for 9 days in DMEM without serum. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control values as follows;

\[
p \geq 0.05 \quad = \text{NS.}
\]
\[
0.05 > p \geq 0.01 \quad = \text{*}
\]
\[
0.01 > p \geq 0.001 \quad = \text{**}
\]
\[
0.001 > p \quad = \text{***}
\]
Fig. 1.7a.

Fig. 1.7b.

Fig. 1.7c.
Fig. 1.7d. Graph representing the loss of sulphated glycosaminoglycans into the medium of explants incubated for 24 hours in DMEM + 20% FCS + 10 unit.ml\(^{-1}\) hyaluronidase and subsequently cultured for a further 8 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control values as follows:

\[
\begin{align*}
p \geq 0.05 & = \text{NS.} \\
0.05 > p \geq 0.01 & = \ast \\
0.01 > p \geq 0.001 & = \ast\ast \\
0.001 > p & = \ast\ast\ast 
\end{align*}
\]

Fig. 1.7e. Graph representing the loss of sulphated glycosaminoglycans into the medium of explants incubated for 24 hours in DMEM + 20% FCS + 20 unit.ml\(^{-1}\) collagenase and subsequently cultured for a further 8 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control values as follows:

\[
\begin{align*}
p \geq 0.05 & = \text{NS.} \\
0.05 > p \geq 0.01 & = \ast \\
0.01 > p \geq 0.001 & = \ast\ast \\
0.001 > p & = \ast\ast\ast 
\end{align*}
\]

Fig. 1.7f. Graph representing the loss of sulphated glycosaminoglycans into the medium of explants incubated in DMEM + 20% FCS + 100 unit.ml\(^{-1}\) collagenase and subsequently cultured for a further 8 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control values as follows:

\[
\begin{align*}
p \geq 0.05 & = \text{NS.} \\
0.05 > p \geq 0.01 & = \ast \\
0.01 > p \geq 0.001 & = \ast\ast \\
0.001 > p & = \ast\ast\ast 
\end{align*}
\]
**Fig. 1.8a.** Graph representing the loss of hydroxyproline (OH-proline) into the medium of explants incubated with enzymes for 24 hours (from Day 0-Day 1) and then cultured for a further 8 days. Each point represents the mean of five replicates.

- □ — Control.
- ● — Cultured without serum.
- ■ — 10 unit.ml⁻¹ hyaluronidase.
- ○ — 20 unit.ml⁻¹ collagenase.
- ▲ — 100 unit.ml⁻¹ collagenase.

For clarity, each graph has been plotted individually in Fig. 1.8b-f. These figures include error bars and an indication of the difference, significant or otherwise between treated and control points.

**Fig. 1.8b.** Graph representing the loss of OH-proline into the medium of explants cultured for 9 days in DMEM + 20% FCS (Control). Error bars indicate the mean ± standard deviation of the mean.

**Fig. 1.8c.** Graph representing the loss of OH-proline into the medium of explants cultured for 9 days in DMEM without serum. Error bars indicate the mean ± standard deviation of the mean. Unpaired student’s t-test values indicate differences from control values as follows:

- \( p \geq 0.05 \) = NS.
- \( 0.05 > p \geq 0.01 \) = *
- \( 0.01 > p \geq 0.001 \) = **
- \( 0.001 > p \) = ***
Fig. 1.8a.

Fig. 1.8b.

Fig. 1.8c.
Fig. 1.8d. Graph representing the loss of OH-proline into the medium of explants incubated for 24 hours in DMEM + 20% FCS + 10 unit.ml⁻¹ hyaluronidase and subsequently cultured for a further 8 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control values as follows:

\[ p \geq 0.05 \quad = \text{NS.} \]
\[ 0.05 > p \geq 0.01 \quad = \ast \]
\[ 0.01 > p \geq 0.001 \quad = \ast\ast \]
\[ 0.001 > p \quad = \ast\ast\ast \]

Fig. 1.8e. Graph representing the loss of OH-proline into the medium of explants incubated for 24 hours in DMEM + 20% FCS + 20 unit.ml⁻¹ collagenase and subsequently cultured for a further 8 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control values as follows:

\[ p \geq 0.05 \quad = \text{NS.} \]
\[ 0.05 > p \geq 0.01 \quad = \ast \]
\[ 0.01 > p \geq 0.001 \quad = \ast\ast \]
\[ 0.001 > p \quad = \ast\ast\ast \]

Fig. 1.8f. Graph representing the loss of OH-proline into the medium of explants incubated in DMEM + 20% FCS + 100 unit.ml⁻¹ collagenase and subsequently cultured for a further 8 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control values as follows:

\[ p \geq 0.05 \quad = \text{NS.} \]
\[ 0.05 > p \geq 0.01 \quad = \ast \]
\[ 0.01 > p \geq 0.001 \quad = \ast\ast \]
\[ 0.001 > p \quad = \ast\ast\ast \]
Fig. 1.8d.

Fig. 1.8e.

Fig. 1.8f.
**Fig. 1.9a.** Graph representing the loss of hyaluronan (HA) into the medium of explants incubated with enzymes for 24 hours (from Day 0-Day 1) and then cultured for a further 8 days. Each point represents the mean of five replicates.

- □ ---- Control.
- –♦—— Cultured without serum.
- –■—— 10 unit.ml⁻¹ hyaluronidase.
- –○—— 20 unit.ml⁻¹ collagenase.
- –▲—— 100 unit.ml⁻¹ collagenase.

For clarity, each graph has been plotted individually in Fig. 1.9b-f. These figures include error bars and an indication of the difference, significant or otherwise between treated and control points.

**Fig. 1.9b.** Graph representing the loss of hyaluronan into the medium of explants cultured for 9 days in DMEM + 20 % FCS (Control). Error bars indicate the mean ± standard deviation of the mean.

**Fig. 1.9c.** Graph representing the loss of hyaluronan into the medium of explants cultured for 9 days in DMEM without serum. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control values as follows:

\[
\begin{align*}
p & \geq 0.05 \quad = \text{NS.} \\
0.05 > p & \geq 0.01 \quad = \ast \\
0.01 > p & \geq 0.001 \quad = \ast\ast \\
0.001 > p & \quad = \ast\ast\ast 
\end{align*}
\]
Fig. 1.9a.

Fig. 1.9b.

Fig. 1.9c.
**Fig. 1.9d.** Graph representing the loss of hyaluronan into the medium of explants incubated for 24 hours in DMEM + 20% FCS + 10 unit.ml⁻¹ hyaluronidase and subsequently cultured for a further 8 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control values as follows:

\[
\begin{align*}
p \geq 0.05 & = \text{NS.} \\
0.05 > p \geq 0.01 & = * \\
0.01 > p \geq 0.001 & = ** \\
0.001 > p & = ***
\end{align*}
\]

**Fig. 1.9e.** Graph representing the loss of hyaluronan into the medium of explants incubated for 24 hours in DMEM + 20% FCS + 20 unit.ml⁻¹ collagenase and subsequently cultured for a further 8 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control values as follows:

\[
\begin{align*}
p \geq 0.05 & = \text{NS.} \\
0.05 > p \geq 0.01 & = * \\
0.01 > p \geq 0.001 & = ** \\
0.001 > p & = ***
\end{align*}
\]

**Fig. 1.9f.** Graph representing the loss of hyaluronan into the medium of explants incubated in DMEM + 20% FCS + 100 unit.ml⁻¹ collagenase and subsequently cultured for a further 8 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control values as follows:

\[
\begin{align*}
p \geq 0.05 & = \text{NS.} \\
0.05 > p \geq 0.01 & = * \\
0.01 > p \geq 0.001 & = ** \\
0.001 > p & = ***
\end{align*}
\]
Fig. 1.9d.

Fig. 1.9e.

Fig. 1.9f.
Discussion.

The object of this first chapter of the thesis was to quantify the effect of enzymic treatment on the loss of matrix components from intact articular cartilage. In order to determine the response to varying concentrations of enzyme, the proportion of total proteoglycans (PG) present in each of the following pools was assessed: 1. PG released into the medium during culture, 2. PG extracted from the tissue in PBS, under associative conditions, 3. PG extracted under dissociative conditions, in 4M guanidine hydrochloride and 4. Non-extractable PG solubilised only with papain. In control cultures the proportion in each fraction (6.8% in the medium, 1.3% in PBS, 47.9% in guanidine and 44% in papain) was similar to previously reported values for intact explants extracted for 24 hours (Poole et al., 1990). Guanidine extraction is believed to depend on diffusion rate and, consequently, on the size of the tissue (Pottenger et al., 1983, 1985). Thus, extracting for a greater length of time or dicing the tissue prior to extraction would increase the guanidine pool and decrease the papain pool. Both enzymes were found to alter the proportion of proteoglycans present in each of the fractions although the mode of action and the effect of increasing enzyme concentrations are different.

Hyaluronidase increased the proportion of PG in both the medium and PBS pools, whilst the guanidine pool was reduced. The non-extractable proportion remained essentially the same as in untreated tissue. Hyaluronidase selectively digests hyaluronan (HA) which forms the central backbone of high molecular weight cartilage aggregates. The increase in PG in the medium and PBS extract may be explained, therefore, as an enzymatic disruption of aggregates to yield PG monomers and aggregates which are small enough to freely diffuse out of the tissue. It is believed that a proportion of tissue PG are non-extractable due to either interactions with collagen or entrapment within the fine collagen network which surrounds the chondrocyte pericellular environment (Campo & Phillips, 1973; Koboyashi & Pedrini, 1973; Campo, 1976, 1981). Collagen has been shown to be unaffected by *Streptomyces* hyaluronidase (Fig. 1.9d) and so it is to be expected that the percentage of non-extractable PG would be unchanged in hyaluronidase-treated explants. It is of interest to note, however, that there is a plateau of response above 10 unit.ml⁻¹ such that higher doses have no additional effect. Peak response releases approximately 35% of the PG into the medium, whilst only a further 4% can be extracted under associative conditions suggesting that a large proportion of PG cannot freely diffuse out of the matrix. This is not a time dependent phenomenon, as explants incubated with hyaluronidase for 48 hr. lost no more PG into the medium than those incubated for 24 hr (10 u.ml⁻¹ for 24 hr lost 32.5 ± 6.9% total PG, 10 u.ml⁻¹ for 48 hr lost 35.8 ± 2.1% total PG. p ≥ 0.05). There may be several possible explanations for this. First, free PG monomers and small aggregates may be produced, but their diffusion rate is insufficient for their removal from the tissue within the time period. This would appear to be unlikely as rolling the tissue in PBS for 24
hr only results in extraction of a further 4% of the total PG. The second possibility is that the enzyme substrate is protected from attack by attached PG. This is a distinct possibility as Faltz et al. (1979) have reported that hyaluronan binding region-link protein complex is able to protect a segment of hyaluronan containing about 50 monosaccharides from attack by \textit{Streptomyces} hyaluronidase. Interactions between PG and molecular species other than hyaluronan, such as collagen, could prevent non-aggregated PG monomer from being released from the tissue. This would, however, represent part of the non-extractable papain pool and so could not explain the proportion of total PG in the guanidine pool in maximally digested tissue (approximately 20%).

Collagenase, by contrast, showed dose-dependent response for all concentrations measured (up to 100 unit.ml\(^{-1}\)). Differences were also evident in the four PG pools. Collagenase, like hyaluronidase, resulted in an increased proportion of PG in both the medium and PBS pools but there was a significant reduction in both the guanidine and papain pools (0.005 < p ≤ 0.01 and 0.01 > p > 0.025 respectively). The majority of cartilage PGs are present as high molecular weight aggregates trapped within a collagenous meshwork. Digestion with collagenase selectively disrupts the collagen architecture and thus allows diffusion out of the tissue of aggregates of a size which normally precludes their loss. As mentioned previously, non-\(4\) extractible PGs are believed to be either associated with collagen or trapped pericellularly within the fine collagenous chondrocyte capsule. Disruption of collagen fibrils may, therefore, destroy collagen-PG interactions, thus allowing loss of this PG pool. Similarly, collagenase-induced pericellular capsule damage may be sufficient to cause diffusion and loss of non-extractable pericellular PG.

As mentioned in the results section, the dosage data presented provide sufficient justification for the use of a single concentration of hyaluronidase (10 unit.ml\(^{-1}\)), but two concentrations of collagenase (20 and 100 unit.ml\(^{-1}\)) future experiments.

In addition to the dose response experiments, time course studies were undertaken to measure the loss of the three major matrix components (PG, collagen and hyaluronan) during nine day culture period. Control explants, cultured in medium containing 20% FCS were compared with tissue cultured in serum-free medium and explants incubated with hyaluronidase or collagenase. Results have been calculated as a percentage of total PG present in the culture medium and, therefore do not give absolute values within the tissue. Several other studies have used a "baseline" value, such as DNA content, wet weight or hydroxyproline and compared proteoglycan levels to this value. This proved to be unfeasible in the present study as no satisfactory baseline value could be used. Cell density varies throughout the tissue and with time and treatment in culture and so measuring on a \(\mu\)g DNA basis would be inappropriate. Similarly, hydroxyproline levels were found to be altered in collagenase-treated tissue, precluding OH-proline content as a baseline. The only value which could be used was wet weight at the start of culture. As cartilage may contain 70%
water this value is likely to be subject to great inaccuracies.

Proteoglycans were lost from control explants at a steady rate of about 10% of the total PG in the tissue per day. It must be appreciated that a constant loss of PG in percentage terms does not necessarily indicate an overall reduction in PG levels within the tissue as it is possible that synthesis of new PG may be sufficient to balance the loss. Results presented elsewhere in this thesis indicate that there is a gradual reduction in safranin-O staining (a PG specific stain) with time in culture, suggesting that an overall decrease in tissue PG levels does occur. The percentage loss of PG measured equates to a half-life of total PG within the tissue of approximately 6.5 days. This value is considerably shorter than PG half-lives measured in vivo which range from 45 days in the young rabbit to 800 days in the human femoral head (Maroudas & Evans, 1974; Maroudas, 1975; Fassbender, 1986; Morales & Hascall, 1989). It has been reported, however, that when transferred to culture conditions there is a general increase in metabolic rate, which may be manifested as heightened levels of degradation of PG (Sokoloff, 1980; Handley et al., 1986). This may, in part, be due to an increase in surface area available for diffusion of PG out of the tissue. There does, however, appear to be an active element to the process as indicated by increased loss during culture at 37°C (6.8% in 24 hours) as compared to extraction in PBS at 4°C (1.3% in 24 hours). The alteration in PG turnover in vitro may be due to several factors. Cartilage in vivo is believed to be in a state which is virtually anaerobic, low in nutrients and growth factors and under constant load. Culture conditions are very different in all these respects and so an alteration in metabolism is to be expected. Depletion has also been identified in the rabbit (Sandy et al., 1978; Nimni, 1982), pig (Tyler, 1985a,b; Ratcliffe et al., 1986) and human explant systems (Verbruggen et al., 1985; Luyten et al., 1987). The rate of loss of tissue PG detected using bovine cartilage explants (10% per day) equates closely to losses measured in the explant systems mentioned above (50% in 3 days in rabbits, 16% in 3 days in pig and 15% in 2 days in human tissue. Previous work utilising bovine cartilage, however, has failed to detect a drop in PG levels during the early stages of culture (Hascall et al., 1983; Campbell et al., 1984; Luyten et al., 1988). The previous studies mentioned measured PG left within the tissue and not PG released into the medium. Such anomalies may, therefore, be due to differences in the assay system used to measure PG depletion and to slight differences in the culture procedures used.

In the absence of serum PGs are lost at an enhanced rate, equating to a half-life of approximately 5 days. The loss of PG from cartilage matrix is believed to be caused primarily by proteolytic cleavage of PG core protein to yield fragments small enough to diffuse out of the matrix. The degradative process is itself controlled by tissue inhibitors which may or may not be active. Serum is believed to contain inhibitors to the proteolytic enzymes responsible for PG degradation (Hirado et al., 1983; Travis & Salvesen, 1983). In the presence of serum, inhibition of tissue protease activity will cause a decrease in the rate of PG degradation and, therefore, PG loss. In addition, PG synthesis is reduced in serum-
free conditions leading to an increased percentage loss of PG from the tissue.

Hyaluronidase-treated cultures lost an increased proportion of tissue PG during enzyme incubation compared to control cultures. This has previously been explained as a disruption of high molecular weight PG-HA aggregates caused by digestion of the HA backbone of the aggregates. Low molecular weight aggregates and PG monomers liberated by such a process are likely to be small enough to diffuse out of the tissue. There is some evidence of residual activity after enzyme incubation, manifested as a slight increase in PG loss above control levels during day 1-3. This may, however, be due to the release of PG liberated during hyaluronidase incubation but not lost from the tissue until after removal of the enzyme. After day 3 PG loss returned to control levels for the rest of the culture period. Similar results were obtained with collagenase-treated explants, with increased PG loss during the enzyme incubation followed by a return to control levels.

The release of OH-proline from control, serum-free and hyaluronidase-treated explants was found to be minimal (approximately 1% in 9 days). No statistically significant difference could be detected between the three treatments. Naturally occurring collagenases are known to exist but their activity in healthy articular cartilage is believed to be very low. Indeed, the turnover rate of collagen in adult cartilage is very slow. Whilst damage may cause small amount of collagen to be released from the cut edges of explants, there is little evidence to suggest an overall increase in collagen catabolism when transferred to culture conditions. Both concentrations of collagenase caused a significant increase in the loss of OH-proline over control levels during incubation and the effect appeared to be dose dependent. Whilst the majority of OH-proline was released during day 0-1, the rate of loss remained above control levels for both concentrations of collagenase throughout the culture period. Whether this is due to residual enzyme activity or simply to the loss of collagen from previously damaged fibrils is unclear.

Control cultures released HA at a similar rate to the loss of PG although the kinetics appear to be different. Whilst PG loss was at a steady rate, the percentage loss of HA increased with time in culture. This is indicative of a response which is not based on an enzyme which cleaves HA. Indeed, HA digesting enzymes have not been reported in adult articular cartilage. It is possible, therefore, that HA loss is linked to PG content within the tissue, such that HA loss is greater when tissue PG content is reduced. This appears entirely feasible as HA forms the central backbone of high molecular weight HA-PG aggregates which are too large to diffuse out the matrix. Cleavage and loss of PG would, therefore, reduce the overall size of aggregates permitting an increase in diffusion out of the tissue. In serum-free conditions the rate of loss of HA was increased over control levels but the shape of the curve remained the same. This may be attributed to the increased loss of PG in the absence of serum and the consequential reduction in the size of aggregates.
Hyaluronidase-treated explants released significantly more HA than control cultures (p < 0.05 at all time points), with the greatest increase occurring during enzyme incubation. Additionally, the total amount of HA detected in the system (tissue and medium) was reduced when measured per mg OH-proline. The assay system used to measure HA will not detect oligosaccharides smaller than 10 monosaccharides whilst the end product of hyaluronidase digestion of HA are hexasaccharides (Christner et al., 1977, 1979; Shimada & Matsumura, 1980). The data presented suggests that a proportion of tissue HA has been digested to a size too small to be detected by the assay. It may be assumed that this pool of highly digested HA fragments would be small enough to be lost from the tissue with ease and consequently the calculated values of percentage HA lost into the medium may be smaller than the actual values.

Collagenase-treatment also increased HA loss above control levels (p < 0.05 for both concentrations at all time point except 14 days for 20 unit.ml⁻¹). Using the same argument as for PG loss, this may be attributed to a loosening of the collagen meshwork, which will allow the loss of aggregates which would normally be retained within the matrix due to their size. The greatest increase over control levels was noted during the first three days of culture and the increase appeared to be dose dependent.

In summary, control cultures showed a constant loss of PG over the culture period with an associated loss of HA. Collagen levels within the tissue appeared little changed. Hyaluronidase treatment increased the loss of both PG and HA without altering collagen catabolism. Collagenase produced an increase in the loss of all three components with the response being dose dependent. The results presented have allowed quantification of the effect of culture and enzyme treatment on the loss of matrix components from articular cartilage. The quantitative approach does not, however, allow investigation of the location of matrix depletion and damage within the tissue, nor does it reveal the more subtle changes in tissue ultrastructure which may occur during culture and enzyme treatment. These questions have been investigated in chapter 2.
CHAPTER 2.

THE MORPHOLOGY AND ULTRASTRUCTURE OF ENZYME-TREATED ARTICULAR CARTILAGE.
Introduction

Articular cartilage is a heterogeneous tissue containing several distinct regions defined by chondrocyte morphology and matrix structure. It is likely, therefore, that damage and response to enzyme treatment will vary throughout the tissue. Classically, cartilage has been divided into several histologically distinct zones according to changes in chondrocyte morphology which occur at different depths in the tissue. The superficial zone is closest to the articulating surface of the cartilage and contains cells which are flattened in a plane parallel to the surface. The chondrocytes in the transition or middle zone are larger more rounded and orientated randomly, whilst deep zone chondrocytes are often arranged in vertical columns, perpendicular to the cartilage surface (Palfrey & Davies, 1966; Weiss et al., 1968; Stockwell, 1979; Ghadially, 1983; Schenk et al., 1986). Ultrastructural differences between surface and deep chondrocytes are also apparent. Deep zone chondrocytes typically contain greater amounts of cytoplasm, more mitochondria and endoplasmic reticulum and have a tendency to accumulate glycogen granules (Ghadially, 1983; Schenk et al., 1986).

Changes in matrix structure and organisation are also apparent with cartilage depth as indicated by biochemical and electron microscopy studies. The size and orientation of collagen fibrils is known to alter throughout cartilage thickness and the changes are believed to be important in cartilage function. Transmission electron microscopy has allowed assessment of fibril size and orientation, whilst scanning electron microscopy has provided evidence concerning three dimensional interrelationships between fibrils. Fibrils in the superficial layer are thinner than those deeper in the tissue and are orientated as a meshwork parallel to the surface. In addition, a region of fine fibrils, termed the lamina splendens has been noted at the actual articulating surface which may act as a protective capsule to the underlying tissue (MacConaill, 1951; Weiss et al., 1968; Meachim & Roy, 1969). Collagen fibrils adjacent to the calcifying tidemark in the deep zone are organised into thick fibres which exhibit periodic banding. The orientation of fibres in the deep zones has been termed "pseudo-random", although the primary direction is perpendicular rather than parallel. The fibres are held in place by being anchored into the underlying calcifying tissue. Within the middle zones, orientation is much more random, becoming increasingly more tangential as the surface is approached (Clarke, 1971; Broom & Marra, 1985; Clark, 1985; Broom, 1988). In the middle and deep zones fine fibrils are present amongst the larger radial fibrils. It has been proposed that these play a role in collagen crosslinking and integrate with the fine pericellular capsule of chondrocytes (Poole et al., 1984, 1987; Clark, 1985). It has recently been proposed that bundles of collagen fibres are further organised into overlapping sheets or plates within the tissue whose orientation is dependent on the loading properties of the joint (Clark, 1990).
Proteoglycan (PG) content is highest in the middle zone, levels in the deep zones are lower and the surface contains the least PG (Stockwell & Scott, 1967; Maroudas et al., 1969; Maroudas, 1979; Franzen et al., 1981; Bayliss et al., 1983). In addition there are qualitative changes in the type and properties of PG from the different zones. The ratio of chondroitin sulphate to keratan sulphate is greatest in the surface zones and decreases with depth. Surface zone PGs are smaller than deep zone PGs, but a greater proportion of surface PGs are capable of forming aggregates with hyaluronan (Franzen et al., 1981; Bayliss et al., 1983). There is also believed to be an increase in dermatan sulphate proteoglycan (DSPG) in the deep zones of articular cartilage.

Whilst surface zone chondrocytes are in direct contact with the interterritorial matrix described above, chondrocytes from the middle and deep zones possess a distinct pericellular matrix termed the chondron (Poole et al., 1984, 1987, 1988c). Each cell is surrounded by a PG rich matrix, which is itself enclosed within a fine collagenous capsule composed of collagens II, VI and IX (Poole et al., 1985; 1988a,b, 1990). The environment directly around chondrocytes may play a regulatory effect on metabolism and so it is of importance to ascertain the effect of both culture and enzyme treatment on this region. It may be highly relevant that collagen VI is believed to be resistant to attack by Clostridium collagenase (Abedin et al., 1982).

The organisation of articular cartilage is highly complex and in explant culture complexity is further enhanced by diffusion effects. Whilst some tissue will be directly adjacent to a diffusion surface other areas will be some distance from it. In order to elicit a response, the enzymes must first diffuse into the tissue. The permeability of cartilage to enzymes will, therefore play a role in the extent and location of enzymically-induced alterations in tissue ultrastructure. Cartilage permeability to large molecules is highly dependent on PG content and so it may be expected that the rate of diffusion at the surface and deep cut edge of the explant will vary (Maroudas, 1975).

In chapter 1, hyaluronidase and collagenase were shown to induce loss of matrix components. The object of this chapter is to determine which areas of the tissue are most affected by the enzymes and to investigate more subtle changes in cartilage morphology and ultrastructure which may be present. In order to study this aspect, cultured cartilage explants were examined by light microscopy and both transmission and scanning electron microscopy.

**Materials and Methods.**

**Light microscopy**

Bovine articular cartilage explants were removed from the metacarpalpalangeal joints of 1-2 year old steers and cultured as described in chapter 1. Control explants were
cultured for up to 14 days, whilst enzyme-treated explants were incubated with Streptomyces hyaluronidase (Sigma, Poole, England), used at 10 unit.ml⁻¹ or Clostridium collagenase (Worthington, Reading, England), used at either 20 or 100 unit.ml⁻¹ for 24 hr at 37°C and subsequently cultured for a further 13 days. After 1, 7 and 14 days in culture, representative explants from each treatment were removed from culture and fixed in 10% formal saline. Subsequent processing was as follows;

Wash in distilled water to remove fixative 2hr.
50% alcohol 30 min.
70% alcohol 30 min.
90% alcohol 30 min.
95% alcohol 30 min.
100% alcohol 3 x 30 min.
Clear in xylene 2 x 30 min.
Impregnate with wax 2 x 1 hr. at 60°C.

Embed in wax, (Stemco 56°C histological wax, Dartford Wax Co, Biggin Hill, England).

Full depth 5μm transverse sections were cut using a spencer 820 microtome and mounted on slides which had previously been degreased by washing in 70% alcohol.

Sections were stained with haematoxylin (BDH, Poole, England) and Safranin-O (Raymond A. Lamb, London, England). Safranin-O is a cationic dye and, therefore, binds to polyanionic proteoglycan molecules in proportion to the amount of negative charge present. The intensity of Safranin-O staining gives a rough indication of the amount of proteoglycan within the tissue (Rosenberg, 1971; Shepard & Mitchell, 1976; Kiviranta et al., 1985). Sections were stained in Ehrlich’s haematoxylin (Humason, 1979) for 15 min. and then differentiated to remove non nuclear staining by washing in 1% acid alcohol (1% 12.5N HCl in 70% alcohol) for approximately 15 sec. The stain was allowed to “blue” by washing the slides in running tap water for 10 min. The sections were then stained in 1% Safranin-O for 2 min, washed briefly in 90% and 100% alcohol, cleared in xylene and mounted using Styrolite mountant (Raymond A. Lamb, London, England). The stained sections were viewed and photographed using an Olympus BH2 photomicroscope.

Transmission electron microscopy

Bovine cartilage explants were removed and cultured as before. On days 1, 7 and 14 representative samples were removed from culture and fixed in 2% glutaraldehyde (TAAB Labs, Reading, England) made up in 0.1M sodium cacodylate buffer (BDH, Poole, England) for 4 hr. Samples were washed in three changes of 0.1M sodium cacodylate buffer and then fixed in 1% osmium tetroxide (TAAB Labs, Reading, England) in 0.1M sodium cacodylate for 90 min. After washing in 0.1M sodium cacodylate, samples were dehydrated, infiltrated and embedded as follows:

70% alcohol 5 min.
1% uranyl acetate in 70% alcohol 20 min.
90% alcohol 2 x 5 min.
96% alcohol 2 x 5 min.
100% alcohol + sodium sulphate 3 x 10 min.
1:1 100% alcohol:Spurrs resin 1 hr.
Spurrs resin overnight.
Spurrs resin 2 x 1 hr.
Embed and polymerise resin 16 hr at 60°C.

Spurr's resin was purchased from Agar Scientific, Stansted, England (Spurr, 1969).

Scanning electron microscopy
Freeze-fractured cartilage samples were prepared using the method reported by Clark (1985). Explants were cultured as before and removed from culture on day 1, 7 and 14. The tissue was washed in 0.1M phosphate buffer (PB) and fixed for 24 hr in 2% glutaraldehyde (TAAB Labs, Reading, England) made up in 0.1M PB. Explants were washed three times in 0.1M PB to remove fixative and then dehydrated in 30%, 50%, 70%, 90% and three changes of 100% alcohol. Freeze fracture was achieved by plunging dehydrated explant samples into liquid N2 for approximately 1 min and then snapping the tissue whilst still immersed in liquid N2. Once fractured the alcohol was replaced with acetone by passing through an acetone sequence (30%, 50%, 70%, 90% and 3 x 100%). Tissue samples were placed in a Polaron critical point dryer and the acetone replaced with liquid CO2. The temperature was raised to above the critical point (approx. 31°C) causing instant drying of the tissue. The cartilage pieces were mounted onto scanning electron microscope stubs (Jeol), and coated with a 20 nm thick layer of gold using a Polaron E500 cool sputter coater. The samples were viewed and photographed using a Jeol JSM 35C scanning electron microscope.

Results

Light microscopy
Photomicrographs of control sections cultured for 1, 7 and 14 days are presented in Fig. 2.1 (pages 58–59). On day 1, sections of cartilage explants showed typical morphology, characterised by flattened cells near to the articular surface of the tissue (Fig. 2.1d.). Deeper in the tissue cells were found to be more rounded and were organised in pairs and sometimes
in columns oriented perpendicular to the articular surface (Fig. 2.1e). Deep zone chondrocytes possessed a pericellular capsule, visualised as an intensely staining halo. Safranin-O staining was intense throughout the matrix but did show variation within the tissue. Staining was greatest in the middle zones and least at the surface. There was a tendency for staining to be greater in the vicinity of chondrocyte columns than in less cellular area of matrix. With time in culture there was a progressive loss in intensity of Safranin-O staining, which was most notable at the surface and at the ends of the explant (Fig. 2.1a,b,c). The use of automatic exposure for photographing sections means that it was often difficult to detect reduced staining in photomicrographs from older cultures. Later in the culture period a small percentage of cells were found to retain an intensely staining halo even in poorly staining areas (Fig. 2.1b). By day 7, cells released from the matrix had formed a monolayer which was visible at the light microscopic level as an area of cells, flattened on to the cut edge of the explant (Fig. 2.1f). This layer rarely exceeded three cells in thickness. Occasionally a thin layer of flattened cells was also visible at the articular surface of the tissue causing a slight roughening of the normally smooth surface. With the exception of the observations already noted there was virtually no alteration in the overall morphology of the tissue between days 1 and 14.

Hyaluronidase-treated cultures fixed on day 1 (directly after enzyme treatment) possessed an area at the surface of the tissue which was virtually unstained with Safranin-O (Fig. 2.2a). The unstained area had a distinct boundary with tissue below which exhibited moderate staining (Fig. 2.2d). In addition, there was often a thin unstained area at the cut edge of the explant. With time, the overall loss of staining seen in control cultures was replicated in hyaluronidase-treated explant, causing a blurring of the distinct boundary noted on day 1 (Fig. 2.2a,b,c). The overall morphology of the tissue and the formation of a monolayer was identical in control and hyaluronidase-treated cultures. Photomicrographs of hyaluronidase-treated explants are presented in Fig. 2.2 (pages, 60-61).

Both collagenase concentrations caused similar histological changes when compared to control sections, with 100 unit.ml⁻¹ producing more notable alterations than 20 unit.ml⁻¹. Photomicrographs of collagenase-treated tissue are presented in Fig. 2.3 (pages 62-65). Changes were most pronounced at the surface and ends of the explant, being visualised as complete disruption of tissue architecture in these areas. Chondrocytes within the disrupted area appeared to be held within an amorphous gel of proteoglycan (Fig. 2.3g). The majority of deep zone tissue appeared to be histologically unaffected by the treatment. The division between highly damaged tissue at the surface and undamaged tissue deeper in the explant was highly defined. In areas where deep tissue was covered by calcified cartilage, matrix damage was minimal, whilst when the calcified layer was absent localised pockets of damage were sometimes apparent. Safranin-O staining was absent in highly disrupted surface and end tissue but remained intense deep in the cartilage. With time in culture, there was a tendency for the amorphous surface layer to be lost and replaced by a covering of cells flattened onto the unaffected tissue below. At the edges of explants, large, highly cellular
nODULES were frequently formed, each one surrounded by a sheath of flattened cells (Fig. 2.3k). Similarly, cellular outgrowths were often apparent at the deep zone cut edge of explants, which were histologically different from the monolayer outgrowths seen in control and hyaluronidase-treated explants in that they were larger, contained rounded as well as flattened cells and were found to stain with Safranin-O (Fig. 2.3i,j). In contrast, staining within the main body of the tissue became progressively less intense with time in a similar fashion to that seen in control and hyaluronidase-treated explants.

**Transmission electron microscopy.**

Electron micrographs of control explants are presented in Fig. 2.4 (pages 66-67) and from hyaluronidase-treated tissue in Fig. 2.5 (pages 68-69). Control explants viewed using transmission electron microscopy showed similar ultrastructural details to those found in previous studies. Surface zone chondrocytes were elongated in a plane tangential to the articular surface of the cartilage (Fig. 2.4a, 2.5a). The nuclei were similarly elongated occupying a large part of the cell. Mitochondria, endoplasmic reticulum and Golgi apparatus were all visible, as were large, non membrane bound lipid deposits. Glycogen granules were rarely seen in surface zone chondrocytes. The outer contour of surface zone cells was, generally speaking, smooth, although small cell processes were visible at the poles and the deep edge of the cell. Chondrocytes deeper in the tissue were larger than surface zone cells and had a more rounded shape (Fig. 2.4b, 2.5b). There appeared to be greater amounts of rough endoplasmic reticulum and the majority of cells contain large quantities of glycogen within the cytoplasm. Cell processes were common in deep zone cells extending into the pericellular matrix.

The matrix near the articular surface of the cartilage was predominantly composed of thin collagen fibrils oriented tangential to the surface (Fig. 2.4c, 2.5c). Whilst proteoglycan granules could be seen estimation of their distribution was difficult using transmission electron microscopy. With depth, there was a progressive increase in the thickness of collagen fibrils and also a change in their orientation (Fig. 2.4d, 2.5d). Deep in the cartilage, many thick fibrils, or fibres were present orientated perpendicular to the articular surface. Thinner fibrils were also apparent amongst the collagen fibres. The matrix around deep zone chondrocytes was found to be different to the main body of interterritorial matrix. Each cell was surrounded by a capsule, composed of tightly packed fine collagen fibrils of similar thickness to those in surface tissue (Fig. 2.4e, 2.5e).

Few morphological changes were apparent with time in culture. As mentioned above it was difficult to quantify PG concentration using electron microscopy and as the main changes over the culture period are associated with PG depletion, it is to be expected that few changes would be seen. The formation of a monolayer at the deep cut edge and, occasionally, at the articular surface of the explant was noted (Fig. 2.4f, 2.5f). Ultrastructurally, this monolayer consisted of highly flattened cells separated from the edge of the explant by an area of fine fibrillar matrix, which showed no obvious orientation.
There was a certain degree of morphological heterogeneity within tissue samples. This accounts for the slight differences in the photomicrographs from control and hyaluronidase-treated explants presented in Figs. 2.4 & 2.5. Overall, however, no differences between hyaluronidase-treated and control tissue were apparent using transmission electron microscopy. This is probably due to the difficulty in assessing PG changes using this system. As a consequence, all of the features mentioned above also apply to hyaluronidase-treated tissue.

Major alterations in both cellular ultrastructure and matrix organisation were seen, however, in collagenase-treated tissue. Electron micrographs of collagenase-treated tissue are presented in Fig. 2.6 (pages 70-73). At the light microscopic level there appeared to be a fine dividing line between areas of total matrix disruption and unaffected areas. Using transmission electron microscopy a further insight into the effect of enzyme action was possible. In the highly disrupted surface zones, virtually all matrix had been destroyed. Small amounts of collagen were noted at the surface of chondrocytes (Fig. 2.6a, b). The cells had a tendency to round up and develop long cellular processes. Intermediate filaments, present in large bundles, were often seen within the cytoplasm of surface zone chondrocytes (Fig. 2.6a, g). At the boundary between highly disrupted tissue and "unaffected matrix" (as assessed using light microscopy), there was a progressive reduction in the thickness and length of collagen fibrils (Fig. 2.6h, i). At their finest, the periodic banding pattern was lost. Even deep within the matrix there appeared to be a reduction in collagen fibril diameter when compared to control matrix (Fig. 2.6j, k). Pericellular alterations within the deep zones of tissue were also noted. The collagen capsule found around chondrocytes was less tightly packed than in control explants, giving the appearance of an expanded 'halo' around the cells. Long cell processes were also noted in deep zone chondrocytes (Fig. 2.6d, f).

The ultrastructural detail of outgrowths formed from collagenase-treated explants was different from the monolayer outgrowths seen in control tissue. The cells had smooth, rounded outlines, often being in direct contact with adjacent cells (Fig. 2.6e). The matrix between outgrowth cells contained highly organised collagen fibrils, oriented parallel to the cell surface (Fig. 2.6i).

Scanning electron microscopy
In some scanning electron microscopy (SEM) studies of articular cartilage, it was found to be necessary to treat samples with hyaluronidase in order to remove PG in order to reveal collagen architecture (Clark, 1985). Using the methods employed in this study, however, PG preservation was not good and no problem was encountered with collagen masking. Changes in hyaluronidase-treated cartilage are primarily due to alterations in PG content, and, therefore, these changes were not apparent using SEM. Scanning electron micrographs of control and hyaluronidase-treated explants appeared identical in this study and have been presented in Fig. 2.7 (pages 74-75).

Freeze-fracture revealed differences between the size and organisation of collagen fibrils throughout the tissue. Near to the surface, collagen fibrils were organised into dense
bundles, with orientation being mainly parallel to the articular surface (Fig. 2.7e). The articular surface was covered by a distinct collagenous ‘mat’, which was often undulating and uneven. It is believed that releasing cartilage from the underlying bone causes curling of the tissue, with the articular surface forming the concave aspect. This may result in unevenness at the articular surface (Ghadially, 1983). Deeper in the tissue, collagen fibrils were arranged into overlapping plates which were clearly visible in freeze-fractured cartilage (Fig. 2.7a, b). Whilst the plates are primarily orientated perpendicular to the articular surface, there is evidence to suggest that they fold over near to the surface to become more parallel (Fig. 2.7b). Using transmission electron microscopy, the orientation of deep zone collagen appeared to be perpendicular to the surface. In the SEM study, however, this was not completely substantiated. Collagen fibrils formed a three-dimensional meshwork and although the overall orientation did appear to be parallel a large number of fibrils did not conform (Fig. 2.7f). This type of arrangement has been termed “pseudo-random” by Broom, (1987).

Surface zone chondrocytes were rarely seen, either because they were masked by collagen or because they had “fallen out” of the tissue during processing leaving an unfilled indentation. Chondrocytes, deeper in the tissue, were more easily visualised and were found to possess a distinct pericellular matrix. Each cell was found to reside within a fine fibrous capsule, the chondron, which had a distinct boundary with the thicker interterritorial matrix surrounding it (Fig. 2.7c, d). Cells typically occupied the entire capsular space. The cells themselves were round in shape and whilst the surface was often roughened, no long cellular processes were seen.

Hyaluronidase-treated explants showed indistinguishable freeze-fracture morphology from control tissue. Changes were apparent, however, in collagenase-treated explants (Fig. 2.8, pages 76-77). In areas where, using light microscopy, only the surface tissue appeared disrupted, it was possible to see alterations throughout the tissue using SEM techniques. The surface was often detached from the rest of the tissue and appeared as a mass of cells and matrix with little organisation (Fig. 2.8). The dividing line between highly-disrupted surface tissue and less-disrupted deep zone tissue appeared to occur at the point where the perpendicular collagen plates, typical of the deeper zones, became more parallel (Fig. 2.8a). In the deep zones the interterritorial matrix was noticeably less three-dimensional and had a “flat” appearance. A reduction in the diameter of the collagen fibrils was also noted (Fig. 2.8f). The pericellular environment was altered, with spaces appearing between the chondrocyte surface and capsule. The capsule itself took on a less fibrous appearance and was expanded, with a less distinct boundary, in comparison with control tissue (Fig. 2.8e). In highly disrupted tissue matrix architecture was completely lost. Chondrocytes were clumped together and surrounded by a fine, disorganised matrix (Fig. 2.8c, d).
Fig. 2.1. The following photomicrographs were prepared from control tissue and stained with haematoxylin and Safranin-O. In all cases the surface of the tissue is toward the top of the page.

**Fig. 2.1a.** Full depth section from a control explant cultured for 1 day. Cells at the surface (S) of the section are flattened, whilst rounded cells organised in columns are present deeper in the tissue (D).

Magnification, x 100.

**Fig. 2.1b.** Full depth section from a control explant cultured for 7 days. There is an overall reduction of staining when compared with Fig. 2.1a above.

Magnification x 100.

**Fig. 2.1c.** Full depth section from a control explant cultured for 14 days. A loss of staining is apparent towards the surface and edge of the tissue, as is a monolayer at the deep margin and articular surface of the explant (M).

Magnification x 100.

**Fig. 2.1d.** Surface tissue from control explant cultured for 1 day. The articular surface of the explant is smooth. Cells are orientated tangentially near the surface and organised into columns deeper in the tissue.

Magnification x 200.

**Fig. 2.1e.** Deep tissue from a control explant cultured for 1 day. The cut edge of the explant in sharply defined.

Magnification x 200.

**Fig. 2.1f.** Deep tissue from a control explant cultured for 14 days. A developing monolayer is visible at the cut edge of the tissue (M).

Magnification x 200.
Fig. 2.2. The following photomicrographs were prepared from explants incubated with 10 unit.ml\(^{-1}\) hyaluronidase and stained with haematoxylin and Safranin-O. In all cases the surface of the tissue is toward the top of the page.

**Fig. 2.2a.** Full depth section from an explant incubated for 1 day with 10 unit.ml\(^{-1}\) hyaluronidase. Staining has been lost from the surface region (S) and shows sharp delineation (arrowheads) with strongly staining tissue below.

Magnification, x 100.

**Fig. 2.2b.** Full depth section from an explant incubated for 1 day with 10 unit.ml\(^{-1}\) hyaluronidase and then cultured for a further 6 days without the enzyme. The unstained surface region is still present although an overall reduction in staining is apparent throughout the tissue, in particular in the deep zones, when compared with Fig. 2.2a.

Magnification x 100.

**Fig. 2.2c.** Full depth section from an explant incubated for 1 day with 10 unit.ml\(^{-1}\) hyaluronidase and then cultured for a further 13 days without the enzyme. The unstained surface region is still present although there is an overall reduction in staining throughout the tissue when compared with Fig. 2.2a.

Magnification x 100.

**Fig. 2.2d.** Surface tissue from an explant incubated for 1 day with 10 unit.ml\(^{-1}\) hyaluronidase. The overall tissue architecture is similar to control explants (Fig. 2.1d) but a clear dividing line between highly stained and poorly staining tissue is evident (arrowheads).

Magnification x 200.

**Fig. 2.2e.** Deep tissue from an explant incubated for 1 day with 10 unit.ml\(^{-1}\) hyaluronidase. The overall tissue architecture is similar to control explant (Fig. 2.1e). The intensity of Safranin-O staining in the deep matrix is also similar to control tissue.

Magnification x 200.

**Fig. 2.2f.** Deep tissue from an explant incubated for 1 day with 10 unit.ml\(^{-1}\) hyaluronidase and then cultured for a further 6 days without the enzyme. Several chondrocytes possess an intensely staining 'halo' localised around the cell (H).

Magnification x 200.
Chapter 2

Fig. 2.2a.

Fig. 2.2b.

Fig. 2.2c.

Fig. 2.2d.

Fig. 2.2e.

Fig. 2.2f.
Fig. 2.3. The following photomicrographs were prepared from explants incubated with 100 unit.ml\(^{-1}\) collagenase and stained with haematoxylin and Safranin-O. Tissue incubated with 20 unit.ml\(^{-1}\) collagenase showed similar changes from control explants to those shown, but to a lesser extent. In all cases the surface of the tissue is toward the top of the page.

**Fig. 2.3a.** Full depth section from an explant incubated for 1 day with 100 unit.ml\(^{-1}\) collagenase. There is extensive tissue disruption to the surface of the explant (S), causing a loss of tissue architecture and staining in this region. There is little apparent damage in the deep tissue (D), which stains normally.

Magnification, x 100.

**Fig. 2.3b.** Full depth section from an explant incubated for 1 day with 100 unit.ml\(^{-1}\) collagenase. There is extensive tissue disruption to the surface of the explant, causing a loss of tissue architecture and staining in this region. Toward the end of the explant disruption is also visible in the deep zones (arrows).

Magnification x 100.

**Fig. 2.3c.** Full depth section from an explant incubated for 1 day with 100 unit.ml\(^{-1}\) collagenase and then cultured for a further 6 days without the enzyme. Tissue disruption is still visible at the surface although the majority of the cells have been lost from this area. A highly cellular outgrowth (O) may be seen at the deep cut edge of the explant, which stains intensely with Safranin-O.

Magnification x 100.

**Fig. 2.3d.** Full depth section from an explant incubated for 1 day with 100 unit.ml\(^{-1}\) collagenase and then cultured for a further 6 days without the enzyme. Tissue disruption is still visible at the surface although the majority of the cells have aggregated in a nodule (N). Disruption to the deep zone tissue can be seen (arrows).

Magnification x 100.

**Fig. 2.3e.** Full depth section from an explant incubated for 1 day with 100 unit.ml\(^{-1}\) collagenase and then cultured for a further 13 days without the enzyme. The overall staining is weaker than in Fig. 2.3a,c. A highly cellular outgrowth (O) may be seen at the deep cut edge of the explant, which stains intensely with Safranin-O.

Magnification x 100.

**Fig. 2.3f.** Full depth section from an explant incubated for 1 day with 100 unit.ml\(^{-1}\) collagenase and then cultured for a further 13 days without the enzyme. A large cellular nodule (N), which stains poorly with Safranin-O is visible at the end of the explant.

Magnification x 100.
Fig. 2.3g. Section from an explant incubated for 1 day with 100 unit.ml⁻¹ collagenase. There is extensive tissue disruption to the surface of the explant, causing a loss of tissue architecture and staining in this region. Chondrocytes released from their matrix have aggregated as a nodule (N).

Magnification, x 200.

Fig. 2.3h. Section from an explant incubated for 1 day with 100 unit.ml⁻¹ collagenase and then cultured for a further 6 days without the enzyme. Deep zone matrix to the left of the picture shows little disruption, whilst loss of matrix architecture is apparent to the right (marked with an asterisk). The darkly stained calcified tissue (C) at the bottom of the picture may prevent enzyme diffusion.

Magnification x 200.

Fig. 2.3i. Section from an explant incubated for 1 day with 100 unit.ml⁻¹ collagenase and then cultured for a further 6 days without the enzyme. A highly cellular outgrowth (O) has developed at the edge of the explant. Cells within the outgrowth tend to be rounded and are surrounded by an intensely staining matrix in contrast to the monolayer outgrowths seen in control cultures (Fig. 2.1f).

Magnification x 200.

Fig. 2.3j. Section from an explant incubated for 1 day with 100 unit.ml⁻¹ collagenase and then cultured for a further 13 days without the enzyme. In this case an outgrowth (O) has completely surrounded an area of calcified tissue (C). Note that the outgrowth is less cellular and stains less intensely than Fig. 2.3i.

Magnification x 200.

Fig. 2.3k. Section from an explant incubated for 1 day with 100 unit.ml⁻¹ collagenase and then cultured for a further 13 days without the enzyme. Cells at the end of the explant have formed into a nodule which shows a distinct morphology. The central area is sparsely populated by cells (marked with an asterisk) but is surrounded by a sheath of flattened cells (arrows). Safranin-O staining is poor throughout.

Magnification x 200.

Fig. 2.3l. Section from an explant incubated for 1 day with 100 unit.ml⁻¹ collagenase and then cultured for a further 6 days without the enzyme. An area of extensive disruption to deep zone matrix is shown (marked with an asterisk). Many of the cells within this area have become elongated and are surrounded by a matrix which stains with Safranin-O.

Magnification x 200.
**Fig. 2.4.** The following electron micrographs were prepared from control tissue. In all cases the direction to the articular surface is indicated by an arrow in the top right corner.

**Fig. 2.4a.** A surface zone cell from cartilage fixed after 1 day in culture. The cell is elongated and contains an elongated nucleus (N), rough endoplasmic reticulum (R) and secondary lysosomes (L). Matrix orientation is parallel to the articular surface. Magnification x 7,400.

**Fig. 2.4b.** Deep zone chondrocytes from cartilage fixed after 1 day in culture. The cells are more rounded than surface chondrocytes (Fig. 2.4a). Glycogen granules (G) can be seen within the cytoplasm of the cell. Matrix orientation is perpendicular to the articular surface and a fine fibrous pericellular matrix may be seen (P). Magnification x 4,100.

**Fig. 2.4c.** Surface zone matrix from cartilage fixed after 14 days in culture. Collagen fibrils show typical periodic banding patterns. Orientation of fibrils is parallel to the articular surface. Magnification x 37,800.

**Fig. 2.4d.** Deep zone matrix from cartilage fixed after 14 days in culture. Collagen fibrils show similar periodic banding patterns to surface zone fibrils (Fig. 2.4c), but are appreciably thicker. There is a fairly large variation in the thickness of fibrils. Orientation is perpendicular to the articular surface. Magnification x 37,800.

**Fig. 2.4e.** Pericellular matrix adjacent to a deep zone chondrocyte from cartilage fixed after 1 day in culture. Matrix near to the cell (C) contains large numbers of proteoglycan granules (arrowheads) and fine collagen fibrils. The thickness of fibrils becomes thicker with distance from the cell. Magnification x 44,300.

**Fig. 2.4f.** Developing monolayer at the deep cut edge of cartilage fixed after 14 days in culture. An elongated cell which has flattened on to the cartilage is clearly visible (C). The cut edge of the tissue is still highly delineated (arrowheads) and separated from the cell by an area of fine fibrous matrix (M). Magnification x 8,600.
Fig. 2.5. The following electronmicrographs were prepared from tissue incubated with 10 unit.ml\(^{-1}\) hyaluronidase for 24 hr and then cultured for up to 13 days. In all cases the direction to the articular surface is indicated by an arrow in the top right corner.

**Fig. 2.5a.** A surface zone cell from cartilage incubated with 10 unit.ml\(^{-1}\) hyaluronidase for 24 hr and fixed directly afterwards. Cellular ultrastructure is similar to the equivalent control cell (Fig. 2.4a). Many cellular processes are visible (arrows). Magnification x 7,400.

**Fig. 2.5b.** Deep zone chondrocytes from cartilage incubated with 10 unit.ml\(^{-1}\) hyaluronidase for 24 hr and fixed directly afterwards. Cellular ultrastructure is similar to the equivalent control cells (Fig. 2.4b). The fine fibrous pericellular matrix is clearly visible (P). Magnification x 4,100.

**Fig. 2.5c.** Surface zone matrix from cartilage incubated with 10 unit.ml\(^{-1}\) hyaluronidase for 24 hr and fixed directly afterwards. Collagen fibrils show typical periodic banding patterns. Orientation of fibrils is tangential to the articular surface. Magnification x 37,800.

**Fig. 2.5d.** Deep zone matrix from cartilage incubated with 10 unit.ml\(^{-1}\) hyaluronidase for 24 hr and cultured for a further 13 days prior to fixation. The size, structure and orientation of collagen fibrils is similar to control explants (Fig. 2.4d). Magnification x 37,800.

**Fig. 2.5e.** Pericellular matrix adjacent to a deep zone chondrocyte from cartilage incubated with 10 unit.ml\(^{-1}\) hyaluronidase for 24 hr and fixed directly afterwards. Magnification x 37,800.

**Fig. 2.5f.** Developing monolayer at the surface of cartilage incubated with 10 unit.ml\(^{-1}\) hyaluronidase for 24 hr and cultured for a further 13 days prior to fixation. An area of fine matrix is visible between the cell and the articular surface of the cartilage (M). Magnification x 4,100.
Fig. 2.6. The following electronmicrographs were prepared from tissue incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and then cultured for up to 13 days. In all cases the direction to the articular surface is indicated by an arrow in the top right corner.

**Fig. 2.6a.** A surface zone cell from cartilage incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. The elongated profile of the chondrocyte has been preserved, but the majority of matrix from around it has been lost. Intermediate filaments (F) are present within the cytoplasm.

Magnification x 5,400.

**Fig. 2.6b.** Middle zone chondrocytes from cartilage incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. Sparcely distributed matrix can be seen as a 'halo' around the cells (H). Many cellular processes are seen projecting from the cells (arrows).

Magnification x 4,100.

**Fig. 2.6c.** Deep zone chondrocytes from cartilage incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. The cells contain large amounts of glycogen and rough endoplasmic reticulum and have numerous cellular processes.

Magnification x 4,100.

**Fig. 2.6d.** Deep zone chondrocyte from cartilage incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. The cell possesses long cellular processes (arrows) and large amounts of rough endoplasmic reticulum.

Magnification x 8,600.

**Fig. 2.6e.** Cellular outgrowth formed from cartilage incubated for 24 hr with 100 unit.ml\(^{-1}\) collagenase and then cultured for a further 13 days prior to fixation. Rounded cells with smooth outlines can be seen separated by a fine, highly organised matrix (M). Two adjacent cells are visible in direct contact with each other (arrows).

Magnification x 3,200.

**Fig. 2.6f.** Deep zone chondrocyte from cartilage incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. Rough endoplasmic reticulum can be seen within the cytoplasm (arrows). The distinction between fine pericellular matrix (P) and coarse interterritorial matrix (M) is particularly clear.

Magnification x 4,100.
Chapter 2.

Fig. 2.6a.

Fig. 2.6b.

Fig. 2.6c.

Fig. 2.6d.

Fig. 2.6e.

Fig. 2.6f.
Fig. 2.6g. Ultrastructural detail of a surface zone cell from cartilage incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. A large area of intermediate filaments is visible (F) within the cytoplasm. Golgi apparatus (G) can also be seen.

Magnification x 30,200.

Fig. 2.6h. Surface zone matrix from cartilage incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and then cultured for a further 13 days prior to fixation. A gradation of collagen disruption from bottom left to top right is apparent. In the more disrupted areas the collagen fibrils are very fine (arrows).

Magnification x 30,200.

Fig. 2.6i. Surface zone matrix from cartilage incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and then cultured for a further 13 days prior to fixation. A gradation of collagen disruption from bottom left to top right is apparent. In the more disrupted areas the collagen fibrils are very fine (arrows).

Magnification x 37,800.

Fig. 2.6j. Disrupted tissue in the deep zone of cartilage incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and then cultured for a further 13 days prior to fixation. The collagen fibrils appear thinner than in control cartilage (Fig. 2.4d). Fine fibrils can be seen at the bottom left of the electronmicrograph (arrows).

Magnification x 37,800.

Fig. 2.6k. Disrupted tissue in the deep zone of cartilage incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and then cultured for a further 13 days prior to fixation. The collagen fibrils appear thinner than in control cartilage (Fig. 2.4d).

Magnification x 37,800.

Fig. 2.6l. Matrix within an outgrowth formed from cartilage incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and then cultured for a further 13 days prior to fixation. The matrix (M) is very fine and oriented in a highly organised way in a plane, parallel to the cell surface (C). Periodic banding can be occasionally visualised.

Magnification x 37,800.
Fig. 2.7. The following scanning electron micrographs were prepared from freeze-fractured samples of articular cartilage. Hyaluronidase-treated explants showed similar detail to control explants and, therefore, they have not been presented separately.

**Fig. 2.7a.** Full-depth freeze-fractured cartilage, fixed on day 1 of culture. Overlapping collagen plates are clearly visible in the deep zones of the tissue (arrows). The overall change of orientation from perpendicular to parallel can be seen as articular surface (S) is approached.

Magnification x 120.

**Fig. 2.7b.** Full-depth freeze-fractured cartilage, fixed on day 1 of culture. The change in orientation of the overlapping collagen plates from perpendicular to parallel can be seen as articular surface (S) is approached (arrows). Columns of chondrocytes are present in the middle and deep zones. The articular surface is uneven in places (arrowheads).

Magnification x 130.

**Fig. 2.7c.** Chondrocyte pair from the deep zone of cartilage fixed on day 1 of culture. One of the chondrocytes has been lost, revealing the fine fibrous inner surface of the chondron capsule (C). There is a distinct boundary between the pericellular and interterritorial matrices (arrows).

Magnification x 2,700.

**Fig. 2.7d.** Chondrocytes in a column from the middle zone of cartilage fixed on day 1 of culture. Note that the chondrocytes completely fill the capsular space and that whilst their surfaces are irregular, no long cellular processes can be seen.

Magnification x 2,700.

**Fig. 2.7e.** Surface zone matrix from cartilage fixed on day 1 of culture. Collagen fibrils are organised into dense bundles, primarily oriented parallel to the articular surface (Arrows).

Magnification x 4,300.

**Fig. 2.7f.** Deep zone matrix from cartilage fixed on day 1 of culture. The collagen is organised into a three-dimensional meshwork. The orientation may be described as "pseudo-random", in that the predominant direction is perpendicular to the articular surface although a large number of fibrils do not follow this orientation. The direction to the articular surface is indicated with an arrow.

Magnification x 4,300.
Fig. 2.8. The following scanning electron micrographs were prepared from freeze-fractured samples of collagenase-treated articular cartilage.

**Fig. 2.8a.** Full-depth freeze-fractured cartilage, incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. The surface of the tissue is highly disrupted (S) and has become detached from the underlying cartilage. The change in disruption pattern appears to be associated with the alteration in orientation of the overlapping collagen plates (arrows).

Magnification x 390.

**Fig. 2.8b.** Surface disrupted tissue from freeze-fractured cartilage, incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. Cells have clumped together and are surrounded by disorganised matrix.

Magnification x 1,000.

**Fig. 2.8c.** Highly disrupted tissue from freeze-fractured cartilage, incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. Cells have clumped together in places and are surrounded by disorganised matrix.

Magnification x 240.

**Fig. 2.8d.** Surface disrupted tissue from freeze-fractured cartilage, incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. Note the disorganised mass of matrix (arrows) and areas of matrix attached to the cell surface (arrowheads)

Magnification x 2,700.

**Fig. 2.8e.** Deep zone tissue from freeze-fractured cartilage, incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. The pericellular capsule (P) appears less fibrous than in control tissue (Fig. 2.7c, d). Spaces are apparent between the chondrocyte surface and the pericellular capsule (arrows) unlike control tissue.

Magnification x 2,700.

**Fig. 2.8f.** Deep zone matrix from freeze-fractured cartilage, incubated with 100 unit.ml\(^{-1}\) collagenase for 24 hr and fixed directly afterwards. The collagen fibrils appear to be thinner than in control tissue (Fig. 2.7f). The matrix meshwork is less three-dimensional, giving it a flat appearance.

Magnification x 4,300.
The object of this chapter was to study the ultrastructure and morphology of bovine articular cartilage, and how it changes when cultured and when treated with enzymes. This has been achieved by using light microscopy and both transmission and scanning electron microscopy. Ultrastructural detail varied from explant to explant and, therefore, general characteristics only will be discussed.

The overall morphology of control explants, fixed directly after culturing was as described in previous studies (Collins, 1949; Ghadially, 1983) and, therefore, will not be discussed in full. Cells near to the articular surface were elongated, flattened parallel to the articular surface, whilst cells deeper in the tissue were more rounded and often arranged in columns. The articular surface itself was often uneven and roughened, a tendency which could be seen using both light and scanning electron microscopy. When cartilage explants are removed without the underlying calcified tissue, as was the case in this study, the cartilage curls so that the straight or slightly convex articular surface becomes concave. At higher magnification the surface shows undulations. The curling of the tissue is caused by the release and subsequent expansion of collagen fibres, normally embedded in the calcified tissue, due to swelling pressure exerted by matrix proteoglycans (Barnett et al., 1963; Ghadially et al., 1982). Artefact, caused by the shrinkage of tissue during processing may also exacerbate this phenomenon. These problems may be overcome by culturing and processing the cartilage still attached to bone and removing bone just prior to sectioning (Ghadially et al., 1982).

With time in culture, few changes in the matrix architecture of the tissue were noted. Cells, released from the tissue, had a tendency to attach and flatten on to the cut edge and form a monolayer. A fine fibrous matrix was formed between the monolayer and cut edge of the explant. The metabolic activity of monolayer cells must be recognised when investigating metabolism in cartilage explants. It is not acceptable, therefore, to assume that DNA or PG synthesis measured in explants is entirely due to cells within the main body of the tissue as is often the case.

During time in culture, there was a progressive reduction in proteoglycan (PG) content, as assessed by Safranin-O staining. The loss of staining was most notable at the surface of the tissue and towards the ends of the explant. These findings are in accordance with the data presented in chapter 1, which indicated a steady loss of glycosaminoglycan from the tissue into the medium of cartilage explants. As explained in the previous chapter, the results from chapter 1 measured the percentage of total glycosaminoglycan present in the medium, and did not give absolute levels within the tissue. The results presented here indicate that a reduction in total tissue PG does indeed occur. PG depletion has also been identified in the rabbit (Sandy et al., 1978; Nimni, 1982), pig (Tyler, 1985a,b; Ratcliffe et
al., 1986) and human explant systems (Verbruggen et al., 1985; Luyten et al., 1987) during the early stages of culture. Previous work using the bovine explant system has suggested that tissue PG remains constant during culture (Hascall et al., 1983; Campbell et al., 1984; Luyten et al., 1988). Such anomalies may be due to slight differences in the culture procedures used. PG’s appear to be preferentially lost from the surface and ends of the tissue. There are several possible explanations for this finding. First, the PG concentration near the surface is less than deeper in the tissue (Stockwell & Scott, 1967; Maroudas et al., 1969; Maroudas, 1979; Franzen et al., 1981; Bayliss et al., 1983). If PGs were lost at the same rate from all areas, this difference would be preserved and could lead to PG levels at the surface which are too low to induce Safranin-O staining, whilst staining would be maintained in the deep zones. Surface tissue aggregates are also believed to be less link-stabilised than deep zone aggregates. Surface aggregates may, therefore, be more susceptible to proteolytic digestion (Muir, 1979). For PG fragments to be lost from the tissue, they must diffuse out of the matrix. The concentration of PG and organisation of collagen fibrils near to the surface of articular cartilage may permit greater levels of PG diffusion from the surface than the deep cut edge.

In hyaluronidase-treated explants, fixed directly after enzyme incubation, Safranin-O staining was obliterated at the surface of the tissue, indicating PG depletion. Staining in the deep zones remained, essentially, normal. The increased loss of glycosaminoglycan in hyaluronidase-treated explants, measured in chapter 1 may be due to loss entirely from the surface matrix. For hyaluronidase to induce PG depletion, it is necessary for the enzyme to digest hyaluronan and, therefore, to disrupt PG-hyaluronan aggregates and form fragments small enough to diffuse out of the matrix. This process relies on the ability of the enzyme to reach the substrate. In this case, hyaluronan is at the centre of large aggregates and attached PG molecules may mask the substrate from the enzyme preventing digestion. A single link-stabilised PG molecule can protect a 50 monosaccharide section of hyaluronan from hyaluronidase attack (Faltz et al., 1979). The spatial pattern of PG monomers along hyaluronan may play an important role in the ability of hyaluronidase to digest hyaluronan. It has been reported that, in the absence of link protein, the number of PG monomers that can bind to hyaluronan is reduced. It is possible that the number and spacing of PG monomers along hyaluronan in the surface zones is reduced, allowing hyaluronidase digestion to occur. There is a greater concentration of PG aggregates in the deep zone and tight packing may prevent hyaluronan digestion. In addition, the rate of diffusion of disrupted aggregates out of the tissue is likely to be greater in the surface zones due to the reduced PG content causing increased pore sizes.

Whilst alterations in the PG content of hyaluronidase-treated tissue were apparent, little change in collagen matrix architecture was seen. The structure of hyaluronidase-treated tissue appeared identical to control tissue using transmission and scanning electron microscopy. PG changes may have been evident using transmission electron microscopy,
but as mentioned in the results section, it was difficult to make qualitative assessments of PG content. Without having undertaken morphometric analysis, it is unwise to draw conclusions on PG content from these studies.

With time in culture hyaluronidase-treated explants showed similar changes to control tissue, with a progressive loss of PG from the surface and cut edge of the explant and the formation of a cellular monolayer.

Collagenase-treated explants showed changes in both the PG content and the collagen architecture of the tissue. Collagen disruption was greatest at the surface where fibrillar organisation was totally destroyed. Diffusion and the ability for the enzyme to gain access to the substrate also appears to play a role. Tissue PG can act to protect collagen from attack by masking the substrate. The variation in PG levels throughout the cartilage may, therefore, be partly responsible for the variation in response of the tissue to collagenase treatment. Whilst the majority of deep tissue showed little disruption at the light microscopic level, areas directly adjacent to the cut edge often showed extensive damage. Collagenase-induced disruption was not, however, seen in deep tissue which was still attached to the underlying calcified cartilage. This is partly due to the reduced ability of the enzyme to diffuse through calcified tissue. The collagen of deep zone cartilage has a tendency to expand when removed from the calcified tissue, increasing the pore size of the tissue and, therefore, permitting greater diffusion.

There was not a gradual reduction in disruption with depth, but rather a highly delineated “cut-off” point which may be due to the structure of the collagen. Using transmission electron microscopy the disruption process could be further elucidated. Near the cut-off point of collagenase response, there was a progressive reduction in collagen fibril thickness, resulting in total destruction near to the surface. It would appear that the primary action of collagenase on collagen fibrils is to disrupt the cross-links which keep the fibril together. Collagenase-treatment results, therefore, in the disruption of large fibrils to release smaller diameter fibrils. This process is progressive, eventually resulting in fibrils and molecules of small enough diameter to be completely degraded by the enzyme. Collagen fibrils in the surface zones of articular cartilage are naturally much thinner than those in the deep zones. Bearing in mind the proposed mode of degradation of collagenase, it is to be expected that total degradation of collagen is much more likely to occur in the surface zones than deeper in the tissue. Indeed, there does appear to be a reduction in the diameter of collagen fibrils in the deep zone, although total disruption was rarely seen. Scanning electron microscopic studies support the theory of selective cross link destruction prior to complete degradation. Within the deep zones the three-dimensional collagen matrix typical of control explants was found to become “flattened” and less three-dimensional in appearance after collagenase-treatment. It can be argued that this is due to the disruption of interfibril crosslinking, which maintains the three-dimensional integrity of the collagen.
These findings are in agreement with Broom (1988) who demonstrated collagenase-induced transformations, involving the breakdown of interfibrillar links, in articular cartilage. In addition, scanning electron microscopy indicated that the "cut-off" point of collagenase disruption was associated with the change in orientation of collagen and collagen plates from radial to tangential. Whether this is significant in the collagenase-induced disruption of collagen architecture is unclear.

Changes were also noted in the ultrastructure of chondrocytes and the pericellular matrix from collagenase-treated cartilage. Intermediate filaments, present in large cytoplasmic bundles, were often seen in surface zone chondrocytes from collagenase-treated cartilage, but were absent in control tissue. Filaments of this type are commonly associated with diseased and damaged tissue (Meachim & Roy, 1967; Zimny & Redler, 1969; Fuller & Ghadially, 1972). The significance of intermediate filaments is not clearly understood, but their distribution suggests an association with degenerative or regressive changes in chondrocytes (Ghadially, 1982). An increase in the numbers and length of cellular processes was noted in cells from all depths and is presumably a response to the altered pericellular environment. In control cartilage the pericellular capsule was composed of tightly packed fibrils and had a distinct boundary with the interterritorial matrix. In contrast, capsules in collagenase-treated cartilage were less fibrous, less tightly packed and appeared to be expanded. The division between pericellular and interterritorial matrix was less well defined. A gap was often evident between cell surface and capsule, caused presumably by either cell shrinkage or capsule expansion. Collagen type VI, a major constituent of the pericellular capsule is known to be resistant to collagenase attack (Abedin, 1982). Alterations are probably caused by disruption of collagens II and IX. Alterations in the pericellular matrix of chondrocytes may be of particular importance in the regulation of cell metabolism as this is the only matrix which is in direct contact with the cell (Poole et al., 1987).

Surface chondrocytes, released from the matrix by collagenase treatment had a tendency to form aggregates or nodules at the ends of the explant. Whilst it is possible that this is an active process, the most plausible explanation is that surface tension during medium changes was sufficient to draw the cells to the end of the tissue. Nodules formed in this way stained poorly with Safranin-O and there was little evidence of matrix secretion. A second type of outgrowth was present in collagenase-treated explants, primarily located at the deep cut edge of the tissue. Outgrowths of this type were very different to the monolayers common in control and hyaluronidase-treated cartilage. Cells within deep zone outgrowths tended to be rounded with smooth profiles. The cells were surrounded by matrix, containing highly organised collagen fibrils, arranged parallel to the cell surface. In addition the matrix stained intensely with Safranin-O, suggesting that proteoglycan synthesis was occurring. Outgrowth cells, which are presumably formed from deep zone chondrocytes are, therefore, highly active in resynthesising matrix. A heterogeneity in the repair potential of surface and deep zone chondrocytes, released by collagenase treatment
is suggested. These findings are in agreement with studies on the synthetic potential of isolated surface and deep chondrocytes in culture (Aydelotte et al., 1988; Siczkowski & Watt, 1990; Archer et al., 1990).

In conclusion, the effect of both hyaluronidase and collagenase on articular cartilage is much more complex than a simple enzyme-substrate relationship. Using biochemical assays to quantify the loss of matrix components in enzyme-treated cartilage only tells part of the story and it is important to recognise much more subtle ultrastructural changes and the location of the effect when investigating alterations in metabolism. The effect of matrix depletion on DNA and proteoglycan synthesis has been investigated in chapters 3 and 4.
CHAPTER 3.
DNA SYNTHESIS IN MATRIX-DEPLETED ARTICULAR CARTILAGE.
Introduction

During development and growth articular chondrocytes, like many other cell types, undergo mitosis to provide for enlargement of the developing epiphysis and articular surface. In many mammalian connective tissues, such as the skin, mitosis remains an important facet of the regulation and turnover of tissue throughout adult life. In contrast, as the animal progresses toward skeletal maturity, chondrocyte division becomes increasingly less common and is absent in healthy mature cartilage (Mankin, 1962a, b, 1963; Rigal, 1962; Pribylova & Hert, 1971; Dustmann et al., 1974a; Puig-Rosado, 1981). Mankin (1964) went on to determine the cell cycle time for articular chondrocytes in immature rabbits as approximately 6 years. This figure is fairly meaningless due to the tiny number of cells involved and is probably based on isolated occurrences rather than a population effect. The results do suggest, however, that once a chondrocyte has divided in the immature rabbit it is unlikely to do so again during the rabbit’s lifespan. It was originally thought that adult articular chondrocytes were terminally differentiated and, therefore, incapable of dividing. This theory has been invalidated by numerous in vitro studies in which isolated chondrocytes regain proliferative potential (Green, 1971; Mohr & Wild, 1977; Webber et al., 1977; Benya & Shaffer, 1982; Adolphe et al., 1983; Sokoloff, 1985). Chondrocyte division has also been reported in various diseased states such as aseptic necrosis of the femoral head and is believed to be the cause of the chondrocytes clusters seen in osteoarthritis (Rothwell & Bentley, 1973; Dustmann et al., 1974b; Hirotani & Ito, 1975). Many workers have reported chondrocyte mitosis in experimentally damaged cartilage and in animal models of arthritis (Telhag, 1972; Hulth, Lindberg & Telhag, 1972; Telhag, 1973; Mayor & Moskalewski, 1974; Hirotani & Ito, 1975; Havdrup & Telhag, 1978; Johnell & Telhag, 1978; Havdrup, 1979; Kunz et al., 1979; Fengler & Franz, 1983). Chondrocytes may, therefore, be said to have left the cell cycle, but retain the potential to re-enter it and divide.

The mammalian cell cycle is broken down into four main phases, G0/1, S, G2 and M phases. G0/1 is generally believed to be a resting phase in the cycle and consequently shows the greatest variation in length. Cells progressing through the cycle leave G0/1 and pass into S-phase during which DNA replication take place. The subsequent G2 period represents the time necessary for the synthesis of various components related to the condensation of chromosomes and organisation of the mitotic spindle, which is finally followed by mitosis during M-phase (Howard & Pelc, 1953; Bright & Gaffney, 1982). Whilst the majority of non-actively dividing cells show G0/1 arrest, it has been reported that certain cells, including chondrocytes may exhibit G2 arrest (Rao, 1980; Ronot et al., 1983, 1985; Moskalewski et al., 1988). The induction of DNA synthesis in quiescent cells can be resolved into two phases termed competence and progression which may be induced by different stimuli (Pledger et al., 1977). It is, therefore, of interest to investigate the particular conditions and factors which maintain quiescence in adult chondrocytes and the changes
which take place in diseased and damaged tissue which allow competence and progression.

For chondrocytes to proliferate several variably related and interacting conditions must be met. These have been described by Sokoloff (1985) as being, 1) attachment and cell shape, 2) the presence of defined growth factors, hormones and other molecular species such as ascorbate and 3) steric hindrance effects caused by the surrounding matrix, which must be overcome. Chondrocytes in intact cartilage show little or no proliferation, whilst chondrocytes maintained in suspension culture show appreciably less proliferation than those attached to a substrate (Sokoloff et al., 1973). When chondrocytes are allowed to attach to a substrate and spread, as in monolayer culture, proliferation may occur provided the other conditions described above are met (Glowacki et al., 1983; Watt, 1988; Watt & Dudhia, 1989). Accompanying the onset of proliferation in monolayer cultures is a loss of differentiated phenotype (Von der Mark, 1986). Macromolecular growth factors and hormones are required as regulators of the competence and progression sequences of the cell cycle. Recently, the effect of the most common growth factors on proliferation in both cartilage explant and cell culture have been investigated. Platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) are believed to act as competence factors for chondrocytes, whilst insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF) and insulin are classically considered progression factors (Pledger et al., 1978; Prins et al., 1982; Osborn et al., 1989). Differences are known to exist between the effect of growth factors on proliferation in cell and explant culture (Sachs et al., 1982; Adolphe et al., 1984; Kato et al., 1987; Froger-Gaillard et al., 1989; Makower et al., 1989; Morales & Hascall, 1989; Osborn et al., 1989) and between immature and mature chondrocytes (Vetter et al., 1985, 1986; Froger-Gaillard et al., 1989). These reported differences may be a consequence of either matrix hindrance effects or receptor expression (Sokoloff, 1985). Indeed, cartilage injury and damage during cell harvesting has been described by Osborn et al., (1989) as a competence factor. An understanding of the changes in chondrocyte responsiveness to growth factors may provide a clue to altered proliferation in diseased tissue.

The object of the present study is to assess the proliferative potential of adult articular chondrocytes under various in vitro conditions. In order to assess matrix hindrance effects, explants of adult articular cartilage have been subjected to enzyme treatment as described and reported in chapters 1 and 2. Tritiated thymidine autoradiography has been employed to visualise cells which have undergone DNA synthesis as it allows both quantification and localisation of any observed effects of matrix depletion. The use of tritiated thymidine is widespread in the study of cell division as it is selectively incorporated into the cell nucleus during S-phase. In addition, the effect of serum (which contains several growth factors in varying concentrations) and purified IGF-1 on chondrocyte proliferation in explant and cell culture has been measured. The use of both monolayer and suspension cultures will allow investigation of cell shape and attachment regulation of cell division.
Materials and Methods

Tritiated Thymidine Labelling

DNA replication was assessed by incubating cultures in medium containing 1 μCi. ml⁻¹ tritiated thymidine ([³H]-TdR) (Amersham International plc, Amersham, England). Incorporation of the nucleotide occurs during S-phase of the cell cycle and so is an indicator of DNA synthesis but not necessarily of cell division. The limitations of this procedure have been reported by Maurer (1981).

The effect of serum and Insulin-like growth factor-1 on [³H]-TdR labelling

Bovine cartilage explants were cultured in DMEM containing 20% (v/v) foetal calf serum (Gibco, Paisley, Scotland), DMEM without serum or DMEM supplemented with 50 ng.ml⁻¹ IGF-1 (a kind gift from Dr. J. Tyler, Strangeways Research Laboratories, Cambridge, England) for 14 days. All medium used contained 1 μCi.ml⁻¹ [³H]-TdR. On days 3, 9 and 14 explants were removed from culture and fixed in 10% formal-saline. After fixation the explants were processed for autoradiography as described below. The percentage of cells, per explant, labelled with the isotope was calculated from the autoradiographs.

The effect of matrix depletion on [³H]-TdR labelling

Explants were set up and their matrix depleted using either hyaluronidase (10 unit.ml⁻¹ DMEM + 20% FCS + 1μCi.ml⁻¹ [³H]-TdR), or collagenase (20 or 100 unit.ml⁻¹ DMEM + 20% FCS + 1μCi.ml⁻¹ [³H]-TdR) as previously described and then cultured for a further 13 days in medium containing 1μCi.ml⁻¹ [³H]-TdR. Explants were fixed and processed for autoradiography on days 1, 3, 5, 7, 9 and 14. The percentage of labelled cells in the surface and deep layers was calculated from the autoradiographs. Additionally, the number of labelled chondrocyte couplets present was determined to give an indication of the amount of cell division occurring within the cartilage. A crude estimation of cell cycle time was attempted by assessing the appearance of labelled chondrocyte couplets within the tissue. A labelled couplet has been defined for these purposes as two (or more) labelled nuclei lying within the same lacuna.

Autoradiography

Explants were removed from culture and fixed in 10% formal-saline. Once fixed they were washed thoroughly in water to remove the fixative and unbound isotope, dehydrated by passing through an alcohol sequence, cleared in xylene and embedded in wax. Sections, 5 μm thick, were cut using a microtome (Spencer 820) and transferred to microscope slides which had previously been degreased by a 70% alcohol wash and coated with 'Histostik' (Histolab, Hemel Hempstead, England). The slides were transferred to the dark room and coated with K2 nuclear research emulsion, obtained in gel form (Ilford Nuclear Research, Mobberley, England). The gel was melted by heating to 43°C and diluted 50:50 with
distilled water prior to use. The coated slides were allowed to dry and then transferred to a light-tight box and exposed at 4°C for 1 week. At the end of this period the slides were returned to the dark room, developed in D19 developer (Kodak, Hemel Hempstead, England), washed and fixed for 5 min in Hypam fixative (Ilford, Mobberley, England). The developing time required for production of autoradiographs with high contrast between background and labelled areas was variable, but normally approximately 5 min 15 sec. To ensure good results a single slide was developed to determine optimum developing time before the rest of the slides were processed. The autoradiographs were counterstained with haematoxylin and safranin-o, dehydrated and mounted. The autoradiographs were viewed using a Zeiss photomicroscope III.

[3H]-Tdr uptake in monolayer and agarose suspension cultures of surface and deep chondrocytes

Monolayer and agarose suspension chondrocyte cultures were set up as follows:

The metacarpalpalangeal joints of steers were obtained and opened as previously described. Slices of cartilage from the surface and deep layers were removed separately, washed briefly in PBS and then cultured as explants for 3 days in DMEM+20%FCS containing twice normal concentrations of antibiotic/mycotic and Gentamycin to combat infection. The extent of surface and deep tissue is indicated in Fig. 3.3a. The cartilage pieces were then finely diced and incubated at 37°C for 30 min in DMEM + 20% FCS + 700 unit.ml⁻¹ pronase (BDH Ltd, Poole, England). The enzymatic supernatant was removed and replaced with DMEM + 20% FCS + collagenase type 1a (Sigma, Poole, England) at the following concentrations:

- Surface zone cartilage: 300 unit.ml⁻¹.
- Deep zone cartilage: 500 unit.ml⁻¹.

The cartilage was rolled for 3 hours at 37°C. Subsequently the supernatant containing digested chondrocytes was removed, spun down at 1000xg, washed twice in DMEM+20%FCS and finally resuspended in 2 ml of medium. The concentration of cells was adjusted to 2.5 x 10⁵ chondrocytes.ml⁻¹ and then cultured as either low density monolayer cultures or suspended over agarose.

Low Density Monolayer Culture: 1 ml chondrocyte suspension was added directly to 35mm tissue culture dishes (Falcon, Cowley, England). The cultures were fed every two days simply by removing the medium and replacing with 1 ml fresh medium.

Agarose Culture: 1 ml aliquots of a 1% solution of molten type V agarose (Sigma, Poole, England) were placed into 35mm tissue culture dishes and allowed to solidify. The agarose was then covered with 1 ml chondrocyte suspension and cultured at 37°C. The cultures were fed by removing the cell suspension and spinning down the cells at 1000 xg. The supernatant was removed and the cells resuspended in fresh medium which was subsequently replaced in the agarose coated dishes.
The chondrocytes were fed every 2 days with DMEM + 20% FCS + 1μCi.ml⁻¹ [³H]-TdR and harvested on days 4 and 8 using the following procedure:

Monolayer; culture medium was removed from the dishes and replaced with 1ml papain solution (see chapter 1). The dishes were placed in a sealable box containing water saturated tissue paper and incubated at 60°C for 16 hr. Excess evaporation was prevented by the saturated environment. At the end of the incubation period the papain digest was flushed round the dish several times using a pasteur pipette to ensure detachment of cells from the plastic dishes and then stored, frozen at -20°C, in a cryotube prior to analysis.

Agarose; culture medium containing chondrocytes was removed from the dish, transferred to a 2ml conical-bottom cryovial (Hughes & Hughes, Romford, England) and spun down at 1000xg. The medium supernatant was removed and the cell pellet stored at -20°C. At the same time 1ml papain solution was added to the coated dish and incubated as described for monolayer cultures. This was to detach any cells which had attached to the agarose. Once removed the papain digest was added to the cell pellet obtained previously, incubated for a further 16 hr. and stored frozen prior to analysis. Uptake of [³H]-TdR was measured as described below.

The effect of serum and IGF-1 on [³H]-TdR uptake in chondrocyte cultures

Monolayer and agarose cultures were set up as described except that full depth tissue was used rather than surface and deep. The cultures were subsequently maintained in medium containing 20% (v/v) FCS, medium without serum or medium supplemented with 50 ng ml⁻¹ IGF-1. All medium contained 1 μCi ml⁻¹ [³H]-TdR. Cells were removed from culture and harvested as above on days 4 and 8. Uptake of [³H]-TdR was measured as described below.

Measurement of [³H]-TdR incorporation in chondrocyte cultures

Tritiated thymidine incorporation into DNA was performed using a modification of the method described by Cottrill (1986). The DNA present in the papain digests was precipitated by adding an equal volume of cold 10% trichloroacetic acid, (TCA) after which the precipitate was removed by filtration across a borosilicate microfibre glass prefiter (Millipore Ltd, Watford, England). The filters were then washed with excess 5% TCA, followed by distilled water and finally 100% alcohol. The filters were transferred to scintillation vials, dried at 60°C and counted in 4 ml. of Scintillator 299 (Canberra-Packard, Pangbourne, England) using a Tricarb 4000 series counter (Packard, Pangbourne, England). Total DNA content was determined as describe below and results expressed as disintigrations per minute (DPM) [³H]-TdR incorporated / μg.DNA.

Measurement of total DNA

Total DNA content in explant and cell cultures was measured using a modification of the method of Royce & Lowther (1979). The method relies on enhanced fluorescence of
ethidium bromide (EBr) as a result of intercalation of the dye into double-helical regions of nucleic acids. Explants or cell cultures were solublised by digestion with papain at 60°C for 24 hr. Reaction mixtures were set up as follows:

Blank: 0.6 ml PBS.

0.2 ml Ribonuclease A (Sigma, Poole, England). Used at 50 μg.ml⁻¹.

Standards: 0.1 ml PBS

0.2 ml Ribonuclease A (Sigma, Poole, England). Used at 50 μg.ml⁻¹.

0.5 ml DNA solution (Sigma, Poole, England).

Concentration range, 0-20 μg.ml⁻¹.

Samples: 0.1 ml PBS.

0.2 ml Ribonuclease A (Sigma, Poole, England). Used at 50 μg.ml⁻¹.

0.5 ml papain digest.

All reaction mixtures were incubated at 37°C for 30 min. to allow complete digestion of double-helical RNA “hairpin loops” and then cooled to 4°C. Two hundred μl. EBr solution, 50 μg.ml⁻¹ in PBS (Sigma, Poole, England) was added to each reaction vial immediately prior to measuring fluorescent emission at 600 nm using a Perkin-Elmer LS-2B filter fluorimeter (Perkin-Elmer Ltd., Beaconsfield, England). DNA concentration in samples was then calculated from the standard curve.

Results

The majority of the work presented in this chapter is based on the quantitative assessment of [³H]-TdR labelled cells, visualised by autoradiography in full depth cartilage explant cultures. Whilst this method was found to have advantages over measuring total [³H]-TdR incorporation, certain problems were encountered. First, explants proved to be highly variable in the number of labelled cells present. Labelled cells were not found evenly throughout the tissue, but had a tendency to be located in groups. Thus, a tissue section which, by chance, intersected several labelled groups would give an abnormally high percentage result whilst abnormally low results were obtained from other sections which missed the labelled groups. This variability was further enhanced by the tiny numbers of cells involved. After 3 days, it would not be unusual to find explants with no labelled cells whilst others contained up to thirty. It is not surprising, therefore, that the standard deviation was usually over 50% of the mean and in some extreme cases actually exceeded mean values. The likelihood of detecting differences between treatments that are statistically significant are accordingly diminished.
With time, there was a tendency for explants to develop outgrowths which formed a continuous monolayer across the culture dish. As with other monolayers, this is formed primarily by cell division and so the majority of outgrowth cells were found to be labelled with [\(^3\)H]-TdR. As the object of the study was to investigate cell division within the main body of the cartilage, labelled outgrowth cells were not included in calculations. Delineating between the main tissue and outgrowth was easy in control and hyaluronidase-treated cultures where cartilage architecture was preserved. In collagenase-treated explants damaged areas existed which were similar in appearance to outgrowths and as a consequence delineation was more difficult. Thus, calculation of results from collagenase-treated explants inevitably included a certain degree of observer-induced estimation.

Whilst acknowledging the limitations of the technique there are advantages over methods measuring total radionucleotide incorporation. It is possible to assess the location of labelled cells within the tissue using autoradiography. This is of particular importance in determining whether heterogeneity of response exists between the different cartilage zones or between damaged and intact tissue. Additionally, using autoradiography it is possible to attempt to remove the effect of labelled cells within the outgrowth, this would be extremely difficult using total [\(^3\)H]-TdR incorporation.

The effect of serum and Insulin-like growth factor-1 on [\(^3\)H]-TdR labelling

Explants were cultured for up to 14 days in DMEM + 20% FCS (control), DMEM (without serum) and DMEM + 50 ng.ml\(^{-1}\) IGF-1. All medium contained 1\(\mu\)Ci.ml\(^{-1}\) [\(^3\)H]-TdR. Fig. 3.1 (pages 94-95) shows the percentage of total cells per explant which were found to be labelled with [\(^3\)H]-TdR as visualised by autoradiography. Control explants were found to contain a small number of labelled cells, predominantly located within the deep zones and generally near to the damaged cut edges of the explant. Explants incubated without serum or with IGF-1 contained significantly fewer labelled cells (0.001 > p for all time points for both treatments), but their location was similar to control tissue.

The effect of enzyme treatment on [\(^3\)H]-TdR labelling:

Explants were incubated for 24 hr in DMEM + 20% FCS (controls) or DMEM + 20% FCS + hyaluronidase (10 unit.ml\(^{-1}\)) or collagenase (20 or 100 unit.ml\(^{-1}\)). All explants were subsequently cultured for up to 13 days in medium without added enzymes. All medium used contained 1\(\mu\)Ci.ml\(^{-1}\) [\(^3\)H]-TdR. Fig. 3.2 (pages 96-99) shows the percentage of total cells per explant which were found to be labelled with [\(^3\)H]-TdR as visualised by autoradiography. Micrographs of autoradiographs prepared from control and enzyme-treated explants are presented in Fig. 3.3 (pages 100-101). In addition, the number of labelled cells present in the surface zones, deep zones and, in collagenase-treated explants, enzymically-damaged tissue were assessed quantitatively. An indication of the extent of tissue designated as surface, deep or damaged is also given in Fig. 3.3. The location of labelled cells within the tissue is presented in Fig. 3.4 (page 102).
Control and hyaluronidase-treated explants were found to contain similar numbers of labelled cells, whilst collagenase-treated tissue exhibited significantly greater numbers of labelled cells (0.05 > p, for both concentrations of collagenase at all time points after day 1). Labelled cell clusters, similar to the type reported in arthritic tissue were never seen in this study. An alteration in the location of labelled cells was also noted in collagenase-treated tissue when compared to controls, although statistical analysis was not possible due to high variability between replicates. In control and hyaluronidase-treated explants the majority of labelled cells were found in the deep zones. In contrast, collagenase-treated explants showed a significant number of labelled cells in the damaged tissue, which is predominantly located at the surface and extremities of the explant. The percentage of cells within the undamaged tissue (both surface and deep) was, consequently, reduced compared to control explants.

Whilst labelled cells were first seen in control autoradiographs on day 1 of culture, labelled couplets were not present within the tissue until day 7 (Fig. 3.5, page 103). Thus a crude estimation of cell cycle time may be calculated as at least 5 days. The lack of labelled cell clusters in this study may, therefore, be a consequence of the long cell cycle.

\[ \text{[H]}^3 \text{TdR uptake in monolayer and agarose suspension cultures of surface and deep chondrocyte} \]

Monolayer and agarose suspension cultures were seeded at a density of 2.5 x 10^5 cells per dish and cultured for 8 days. Photomicrographs of cultures after 4 and 8 days are presented in Fig. 3.6 (pages 104-105). The cells in monolayer culture attached and flattened during the first day of culture and subsequent growth resulted in the formation of a confluent monolayer after approximately 5-6 days. Surface zone chondrocytes appeared to grow at a slightly slower rate than deep zone chondrocytes so that after 8 days the "confluent" monolayer was incomplete in places. When cultured in suspension over agarose, cells clumped and formed clusters during the first few days of culture. The cells remained rounded until day 4 of culture when flattened cells began to appear on the surface of the clusters and attached to the agarose. The number of flattened cells increased with time after this point. Whorled clusters in which a central core of rounded cells was surrounded by flattened cells were particularly apparent after 8 days in culture. Surface zone chondrocytes appeared to flatten less than the equivalent deep zone chondrocyte cultures.

Monolayer and agarose suspension cultures prepared from surface or deep chondrocytes were labelled in medium containing 1µCi.ml-1 [\text{[H]}^3 \text{TdR for up to 8 days. Fig. 3.7 (page 106) indicates the uptake of the radioisotope into chondrocytes, measured as disintegrations per minute (DPM) [\text{[H]}^3 \text{TdR incorporated per µg total DNA in the culture. Statistical treatment of this data is presented in Fig. 3.8 (page 107). Monolayer cultures were found to incorporate significantly more [\text{[H]}^3 \text{TdR than agarose cultures (p < 0.05) for both surface and deep zone chondrocytes. The difference was most marked at 4 days. The in-}
The rate of incorporation in monolayer cultures was found to drop off between 4 and 8 days. The rate in agarose suspension cultures picked up over this period as compared to day 0-4.

In all cases deep zone cultures incorporated more [3H]-TdR than the equivalent surface zone cultures. This difference was not found to be significant (p > 0.05) and so this may be viewed as a trend only.

The effect of serum and IGF-1 on [3H]-TdR uptake in chondrocyte cultures

Full depth chondrocytes were cultured as a monolayer or as a suspension over agarose in DMEM + 20% FCS, DMEM (without serum) or DMEM + 50ng.ml⁻¹ IGF-1 for up to 8 days. Photomicrographs of cultures after 4 and 8 days are presented in Fig. 3.9 (pages 108-111). Chondrocytes cultured with serum appeared very similar to deep zone cultures as described above. Cells cultured without serum or with IGF-1 were markedly different to serum-containing cultures, but were very similar to each other. The vast majority of chondrocytes failed to flatten in monolayer culture in the absence of serum and their numbers appeared to change little throughout the culture period. Similarly, chondrocytes remained rounded in suspension over agarose and failed to form the huge clusters so common in serum-containing cultures.

The uptake of [3H]-TdR in chondrocytes cultured in the presence or absence of serum and IGF-1 is presented in Fig. 3.10 (page 112), with statistical analysis in Fig. 3.11 (page 113). Serum-containing cultures incorporated [3H]-TdR at a rate similar to that of the deep zone cultures described above. Chondrocytes cultured without serum or in the presence of IGF-1 incorporated significantly less of the radionucleotide than serum containing cultures (p < 0.05). Whilst monolayer cultures always incorporated more [3H]-TdR than the equivalent agarose suspension culture, no statistically significant difference was detected between the four serum-free and IGF-1 treatments investigated. This may be a consequence of the low levels of incorporation detected in these cultures.
Fig. 3.1a. Graph representing the percentage of total cells per autoradiograph labelled with [3H]-TdR. Explants were cultured in medium supplemented with 20% (v/v) FCS, medium alone or medium supplemented with 50 ng.ml⁻¹ IGF-I. Each point represents the mean of at least six replicates.

- □ DMEM + 20% (v/v) FCS.
- ■ DMEM (without serum).
- ▲ DMEM + 50 ng.ml⁻¹ IGF-I.

For clarity, each graph has been plotted individually in Fig. 3.1b-d. These figures include error bars and an indication of the difference, significant or otherwise between control medium (DMEM + 20% FCS) and other treatments.

Fig. 3.1b. Graph representing the percentage of total cells labelled with [3H]-TdR per autoradiograph prepared from explants cultured in DMEM + 20% FCS. Error bars indicate the mean ± standard deviation of the mean.
Fig. 3.1c. Graph representing the percentage of total cells labelled with [\textsuperscript{3}H]-TdR per autoradiograph prepared from explants cultured in DMEM (without serum). Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control medium (DMEM + 20% FCS) as follows;
\[ p \geq 0.05 = \text{NS.} \]
\[ 0.05 > p \geq 0.01 = * \]
\[ 0.01 > p \geq 0.001 = ** \]
\[ 0.001 > p = *** \]

Fig. 3.1d. Graph representing the percentage of total cells labelled with [\textsuperscript{3}H]-TdR per autoradiograph prepared from explants cultured in DMEM + 50 ng.ml\textsuperscript{-1} IGF-I. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control medium (DMEM + 20% FCS) as follows;
\[ p \geq 0.05 = \text{NS.} \]
\[ 0.05 > p \geq 0.01 = * \]
\[ 0.01 > p \geq 0.001 = ** \]
\[ 0.001 > p = *** \]
Fig. 3.2a. Graph representing the percentage of total cells labelled with [3H]-Tdr per autoradiograph prepared from explants incubated with enzymes for 24 hours (from Day 0-Day 1) and then cultured for a further 13 days. Each point represents the mean value of at least twelve replicates.

- Control.
- 10 unit.ml\(^{-1}\) Hyaluronidase.
- 20 unit.ml\(^{-1}\) Collagenase.
- 100 unit.ml\(^{-1}\) Collagenase.

For clarity, each graph has been plotted individually in Fig. 3.2b-e. These figures include error bars and an indication of the difference, significant or otherwise between treated and control points.

Fig. 3.2b. Graph representing the percentage of total cells labelled with [3H]-Tdr per autoradiograph prepared from explants cultured for 14 days in DMEM + 20% FCS (Control). Error bars indicate the mean ± standard deviation of the mean.

Fig. 3.2c. Graph representing the percentage of total cells labelled with [3H]-Tdr per autoradiograph prepared from explants incubated for 24 hours in DMEM + 20% FCS + 10 unit.ml\(^{-1}\) Hyaluronidase and subsequently cultured for a further 13 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control as follows;

\[ p \geq 0.05 = \text{NS.} \]
\[ 0.05 > p \geq 0.01 = * \]
\[ 0.01 > p \geq 0.001 = ** \]
\[ 0.001 > p = *** \]
Fig. 3.2a.

Fig. 3.2b.

Fig. 3.2c.
**Fig. 3.2d.** Graph representing the percentage of total cells labelled with $[^3]H$-TdR per autoradiograph prepared from explants incubated for 24 hours in DMEM + 20% FCS + 20 unit.ml$^{-1}$ Collagenase and subsequently cultured for a further 13 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control as follows; 
\[
\begin{align*}
p \geq 0.05 & = \text{NS.} \\
0.05 > p \geq 0.01 & = * \\
0.01 > p \geq 0.001 & = ** \\
0.001 > p & = ***
\end{align*}
\]

**Fig. 3.2e.** Graph representing the percentage of total cells labelled with $[^3]H$-TdR per autoradiograph prepared from explants incubated for 24 hours in DMEM + 20% FCS + 100 unit.ml$^{-1}$ Collagenase and subsequently cultured for a further 13 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control as follows; 
\[
\begin{align*}
p \geq 0.05 & = \text{NS.} \\
0.05 > p \geq 0.01 & = * \\
0.01 > p \geq 0.001 & = ** \\
0.001 > p & = ***
\end{align*}
\]
Fig. 3.2d.

% Total Cells labelled with [3H]-TdR.

Time/Days

Fig. 3.2e.

% Total Cells Labelled with [3H]-TdR.

Time/Days
Fig. 3.3. The following figures are photomicrographs of [3H]-TdR autoradiographs indicating cells which have undergone DNA replication. Labelled cells appear black as they are covered by silver grains. Unless stated, sections have been counterstained with haematoxylin. In all cases the surface of the tissue is towards the top of the page.

Fig. 3.3a. Control section stained with haematoxylin and Safranin-O. The extent of surface (S) and deep (D) tissue indicated is as used in localisation studies of labelled cells and in the preparation of chondrocyte cultures from surface and deep tissue.
Magnification x 100.

Fig. 3.3b. Autoradiograph prepared from a control explant cultured for 9 days in medium containing [3H]-TdR. A group of labelled cells (arrows) may be seen near to the deep cut edge of the explant.
Magnification x 200.

Fig. 3.3c. Autoradiograph prepared from a control explant cultured for 9 days in medium containing [3H]-TdR. A single labelled cell is present within the tissue. A monolayer has formed at the edge of the explant and contains exclusively labelled cells (M). Inset shows a labelled couplet in which two labelled nuclei reside within the same lacuna.
Magnification x 200.

Fig. 3.3d. Autoradiograph prepared from an explant incubated with 100 unit.ml-1 collagenase and then cultured for a further 8 days. All medium contained [3H]-TdR. The disrupted tissue at both the surface and deep margins of the explant contain large numbers of labelled cells. There are very few labelled cells in the matrix which appears undisturbed.
Magnification x 100.

Fig. 3.3e. Autoradiograph prepared from an explant incubated with 100 unit.ml-1 collagenase and then cultured for a further 8 days. All medium contained [3H]-TdR. There is an area of tissue disruption (D) in the deep zones which contains large numbers of labelled cells. Additionally, labelled cells are present where the surface tissue has been damaged. A nodule (N) has formed at the end of the explant which contains labelled cells only within the flattened sheath surrounding it (arrows).
Magnification x 100.

Fig. 3.3f. Autoradiograph prepared from an explant incubated with 100 unit.ml-1 collagenase and then cultured for a further 8 days. All medium contained [3H]-TdR. Labelled cells are present where tissue disruption is apparent, but are absent where matrix architecture is preserved.
Magnification x 200.
**Fig. 3.4.** The location of $[^3]H$-TdR labelled cells in cartilage explants treated with enzymes as indicated. The figures represent the percentage of total labelled cells per explant present in each of the areas listed as defined in Fig. 3.3. In control and hyaluronidase-treated explants, values in the surface and deep zones of the tissue have been presented. Collagenase-treated explants contained areas where the tissue architecture had been enzymically disrupted and so an additional value (disrupted tissue) has been included. Due to the lack of visible tissue disruption in control or hyaluronidase-treated explants a disrupted tissue value could not be calculated and is, therefore, not applicable (NA). Each value represents the mean of at least four replicates. Statistical analysis has not been attempted on this data as the variability within groups was particularly large.
Fig. 3.5. The appearance of [3H]-TdR labelled cells (Fig. 3.5a) and labelled couplets (Fig. 3.5b) within articular cartilage cultured in DMEM + 20% FCS for up to 14 days. A labelled couplet has been defined as two labelled nuclei residing within the same pericellular lacuna.
Fig. 3.6. The following figures are photomicrographs of surface or deep zone chondrocytes cultured as a monolayer on tissue culture plastic or in suspension over agarose.

**Fig. 3.6a.** Deep zone chondrocytes cultured as a monolayer on tissue culture plastic for 4 days. The majority of cells have attached and flattened and are approaching confluence. Most cells have an elongated or bipolar morphology. Magnification x 200.

**Fig. 3.6b.** Deep zone chondrocytes cultured as a monolayer on tissue culture plastic for 8 days. A confluent monolayer has been formed. Magnification x 200.

**Fig. 3.6c.** Surface zone chondrocytes cultured as a monolayer on tissue culture plastic for 4 days. Cells are much more sparsely populated than in the equivalent deep zone culture (Fig. 3.6a). The cells have a more polygonal morphology than deep zone chondrocytes. Magnification x 200.

**Fig. 3.6d.** Surface zone chondrocytes cultured as a monolayer on tissue culture plastic for 8 days. The chondrocytes have failed to reach confluence unlike the equivalent deep zone culture (Fig. 3.6b). Cells have spread to a much greater extent. Magnification x 200.

**Fig. 3.6e.** Deep zone chondrocytes cultured in suspension over agarose for 4 days. Chondrocytes have formed aggregates, some of which have attached to the agarose and are beginning to spread on it. Magnification x 200.

**Fig. 3.6f.** Deep zone chondrocytes cultured in suspension over agarose for 8 days. Some cells have remained rounded, but the majority have flattened to form a monolayer on the agarose. Magnification x 200.

**Fig. 3.6g.** Surface zone chondrocytes cultured in suspension over agarose for 4 days. Chondrocytes have formed aggregates but there is little evidence of attachment and flattening on the agarose, unlike in deep zone cultures (Fig. 3.6e). Magnification x 200.

**Fig. 3.6h.** Surface zone chondrocytes cultured in suspension over agarose for 8 days. Cellular aggregates have attached and begun to spread on the agarose, but not to the same extent as deep zone chondrocytes (Fig. 3.6f). Magnification x 200.
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Fig. 3.6a. Fig. 3.6b.

Fig. 3.6c. Fig. 3.6d.

Fig. 3.6e. Fig. 3.6f.

Fig. 3.6g. Fig. 3.6h.

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Fig. 3.7. The uptake of $[^3]H$-TdR by surface and deep chondrocytes cultured as either monolayer or over agarose in DMEM + 20% FCS. Uptake has been measured as disintegrations per minute (DPM) incorporated per μg DNA. Each column represents the mean of four replicates, error bars indicate the standard deviation.

- ■ Deep zone chondrocytes cultured as a monolayer.
- □ Surface zone chondrocytes cultured as a monolayer.
- ■ Deep zone chondrocytes cultured over agarose.
- □ Surface zone chondrocytes cultured over agarose.

Statistical analysis of the data, including analysis of variance and Newman-Keuls multiple comparison tests are presented in Fig. 3.8 (page 107).
Fig. 3.8. Statistical analysis used to compare the uptake of [³H]-TdR in surface and deep chondrocytes cultured as monolayers or over agarose in DMEM + 20% FCS. Tabulated results of one way, four group, analysis of variance tests are presented in Fig. 3.8a & b. A p-value of less than 0.05 has been taken to indicate a significant difference between the groups. Newman-Keuls multiple comparison tests have been used to determine differences between treatment groups (Fig. 3.8c & d). In both cases treatment means have been ranked in ascending order using the following abbreviations:

- Deep zone chondrocytes cultured as a monolayer.  
- Deep zone chondrocytes cultured over agarose.  
- Surface zone chondrocytes cultured as a monolayer.  
- Surface zone chondrocytes cultured over agarose.

NS indicates that the difference between means was not significant at the 5% level, whilst * indicates that the difference was significant at the 5% level.
Fig. 3.9. The following figures are photomicrographs of full depth chondrocytes cultured as a monolayer on tissue culture plastic or in suspension over agarose in DMEM + 20% FCS, DMEM (without serum) or DMEM + 50 ng.ml⁻¹ IGF-1.

**Fig. 3.9a.** Chondrocytes cultured as a monolayer on tissue culture plastic for 4 days in DMEM + 20% FCS. The majority of cells have attached and spread and formed a monolayer which has nearly reached confluence.

Magnification x 200.

**Fig. 3.9b.** Chondrocytes cultured as a monolayer on tissue culture plastic for 8 days in DMEM + 20% FCS. A confluent monolayer has been formed.

Magnification x 200.

**Fig. 3.9c.** Chondrocytes cultured as a monolayer on tissue culture plastic for 4 days in DMEM (without serum). Most of the cells have failed to attach and spread on the tissue culture plastic and have retained their rounded morphology.

Magnification x 200.

**Fig. 3.9d.** Chondrocytes cultured as a monolayer on tissue culture plastic for 8 days in DMEM (without serum). More cells have spread than after 4 days (Fig. 3.9c), but the majority remain rounded.

Magnification x 200.

**Fig. 3.9e.** Chondrocytes cultured as a monolayer on tissue culture plastic for 4 days in DMEM + 50 ng.ml⁻¹ IGF-1. Whilst some of the cells have attached, the majority have failed to spread on the tissue culture plastic and have retained their rounded morphology. There is little difference between this culture and the equivalent serum-free culture (Fig. 3.9c).

Magnification x 200.

**Fig. 3.9f.** Chondrocytes cultured as a monolayer on tissue culture plastic for 8 days in DMEM + 50 ng.ml⁻¹ IGF-1. More cells have spread than after 4 days (Fig. 3.9e), but the majority remain rounded. There appears to be a slightly greater propensity for chondrocytes to spread in this culture than in the equivalent serum-free culture (Fig. 3.9d).

Magnification x 200.
Fig. 3.9g. Chondrocytes cultured in suspension over agarose for 4 days in DMEM + 20% FCS. Multicellular aggregates have formed, some of which have attached and begun to spread on the agarose.

Magnification x 200.

Fig. 3.9h. Chondrocytes cultured in suspension over agarose for 8 days in DMEM + 20% FCS. A large proportion of the cells have attached and spread on the agarose forming a monolayer which contains clumps of rounded cells within it.

Magnification x 200.

Fig. 3.9i. Chondrocytes cultured in suspension over agarose for 4 days in DMEM (without serum). The cells have failed to aggregate and have retained their rounded morphology.

Magnification x 200.

Fig. 3.9j. Chondrocytes cultured in suspension over agarose for 8 days in DMEM (without serum). Whilst the vast majority of cells have retained their rounded morphology, a small proportion have attached and begun to spread on the agarose.

Magnification x 200.

Fig. 3.9k. Chondrocytes cultured in suspension over agarose for 4 days in DMEM + 50 ng.ml⁻¹ IGF-1. The cells have failed to aggregate and have retained their rounded morphology. There is little difference between this culture and the equivalent serum-free culture (Fig. 3.9i).

Magnification x 200.

Fig. 3.9l. Chondrocytes cultured in suspension over agarose for 8 days in DMEM + 50 ng.ml⁻¹ IGF-1. Whilst the vast majority of cells have retained their rounded morphology, a small proportion have attached and begun to spread on the agarose. There is little difference between this culture and the equivalent serum-free culture (Fig. 3.9j).

Magnification x 200.
Fig. 3.9g.

Fig. 3.9h.

Fig. 3.9i.

Fig. 3.9j.

Fig. 3.9k.

Fig. 3.9l.
Fig. 3.10. The uptake of [³H]-TdR by chondrocytes cultured as either monolayer or over agarose in DMEM + 20% FCS, DMEM (without serum) or DMEM+ 50 ng.ml⁻¹ IGF-1. Uptake has been measured as disintegrations per minute (DPM) incorporated per μg DNA. Each column represents the mean of three replicates, error bars indicate the standard deviation.

- ■ Cultured as a monolayer in DMEM + 20% FCS.
- □ Cultured over agarose in DMEM + 20% FCS.
- ■ Cultured as a monolayer in DMEM + 50 ng.ml⁻¹ IGF-1.
- □ Cultured over agarose in DMEM + 50 ng.ml⁻¹ IGF-1.
- ■■ Cultured as a monolayer in DMEM (without serum).
- □□ Cultured over agarose in DMEM (without serum).

Statistical analysis of the data, including analysis of variance and Newman-Keuls multiple comparison tests are presented in Fig. 3.11 (page 113).
**Fig. 3.11a. 4 Days.**

**Fig. 3.11b. 8 Days.**

**Fig. 3.11c. 4 Days.**

**Fig. 3.11d. 8 Days.**

**Fig. 3.11.** Statistical analysis used to compare the uptake of $[^3]$H]-TdR in chondrocytes cultured as monolayers or over agarose, in DMEM + 20% FCS, DMEM without serum or DMEM + 50 ng.ml$^{-1}$ IGF-1. Tabulated results of one way, six group, analysis of variance tests are presented in Fig. 3.11a & b. A p-value of less than 0.05 has been taken to indicate a significant difference between the groups. Newman-Keuls multiple comparison tests have been used to determine differences between treatment groups (Fig. 3.11c &d). In both cases treatment means have been ranked in ascending order using the following abbreviations:

- Cultured as a monolayer in DMEM + 20% FCS: Ser M.
- Cultured over agarose in DMEM + 20% FCS: Ser A.
- Cultured as a monolayer in DMEM (without serum): NS M.
- Cultured over agarose in DMEM (without serum): NS A.
- Cultured as a monolayer in DMEM + 50 ng.ml$^{-1}$ IGF-1: IGF M.
- Cultured over agarose in DMEM + 50 ng.ml$^{-1}$ IGF-1: IGF A.

NS indicates that the difference between means was not significant at the 5% level, whilst * indicates that the difference was significant at the 5% level.
Discussion.

The object of this chapter was to investigate the control of cell division in cultures of articular cartilage. All the experiments relied, however, on the measurement of $[^3H] \text{-TdR}$ incorporation and, as such, indicated cells which have passed through S-phase of the cell cycle but not necessarily through mitosis. Whilst in the majority of cases DNA replication is followed by cell division, G2 arrest is not unknown and has been reported in chondrocytes (Rao, 1980; Ronot et al., 1983, 1985; Moskalewski et al., 1988). This distinction must, therefore, be recognised when drawing conclusions from such studies.

Full depth explants of bovine articular cartilage cultured in DMEM + 20% FCS showed low but detectable levels of DNA synthesis. The number of labelled cells within the tissue increased in a linear fashion during the culture period such that after 14 days in culture approximately 5% of the total chondrocytes had undergone DNA replication. In addition, cells released from the tissue had a tendency to flatten onto the surface of the cartilage forming an outgrowth and, eventually, a confluent monolayer across the culture dish. Autoradiography revealed that the vast majority of cells in the outgrowth were labelled with $[^3H] \text{-TdR}$, suggesting that matrix hindrance and/or cell shape may play a role in the regulation of DNA synthesis. Autoradiography also allowed localisation of labelled cells within the cartilage and has shown that the majority of labelled cells were found in the deep zones adjacent to the cut edge of the explant. A certain degree of heterogeneity of response was detected between different tissue samples. Some explants were found to contain large numbers of labelled cells whilst others contained none. Similarly, although the majority of labelled cells were found in a band near to the subchondral bone, their distribution within this band was not random. Labelled cells tended to be found in groups and whilst there appeared to be more toward the ends of sections, no other correlation could be made between location and matrix damage or depletion in control explants.

It is not clear, however, whether the levels of DNA synthesis detected reflect activity present in vivo or are a consequence of transfer to culture conditions. Cell division is believed to occur during the development of articular cartilage but becomes increasingly less common with age and is absent in the adult. The last dividing cells found during development are present near to the subchondral bone (Mankin, 1962a, b, 1963; Rigal, 1962; Pribylova & Hert, 1971). It is possible, therefore, that 1-2 year-old cattle have not reached skeletal maturity and that chondrocyte division is still present. The location of labelled cells in a band near to the subchondral bone appears to support this idea. The location of labelled cells was also near to the cut edge of the explant and DNA synthesis may be due to matrix damage in this area or to alteration in the environmental conditions in this area. In order to determine which theory is correct it would be necessary to measure $[^3H] \text{-TdR}$ in vivo. This would, of course, not be feasible considering the source of the cartilage.
In explant culture, far more deep zone chondrocytes were found to incorporate \[^3\text{H}\]TdR than surface cells. In order to study whether this heterogeneity was due to innate differences between the cells or caused by environmental conditions, chondrocytes from the two zones were isolated and cultured. The effect of different culture conditions on \[^3\text{H}\]TdR incorporation has been discussed in detail below. Whilst deep zone cells were found to incorporate more \[^3\text{H}\]TdR than surface zone cells in both monolayer and agarose suspension culture, the difference was not statistically significant. The small number of replicates employed in the study and the fact that both surface and deep cultures may contain similar middle zone cells may explain the lack of statistical significance. Morphological differences were apparent, however, suggesting that deep zone chondrocytes had a greater ability to proliferate, attach and spread on both agarose and tissue culture plastic. Deep zones monolayer cultures reached confluence more rapidly than surface zone cultures. Over agarose, deep zone chondrocytes attached and spread, in contrast to surface zone cells. Previous reports have also suggested that deep zone chondrocytes from bovine articular cartilage have a greater proliferative potential than surface zone cells when cultured either embedded in soft agarose or as a monolayer (Aydelotte & Kuettner, 1988; Siczowski & Watt, 1990). There is, therefore, a certain degree of evidence to suggest that the proliferative heterogeneity of chondrocytes in tissue is retained when isolated.

DNA synthesis in cartilage explants was found to be dependent on serum. Explants cultured in DMEM or in DMEM + 50 ng.ml\(^{-1}\) IGF-1 contained significantly less labelled cells than those cultured in DMEM + 20\% FCS (p < 0.001 for both treatments at all time points). Without serum, labelled cells were extremely rare and their numbers did not increase after day 3. Serum was only removed from the system immediately prior to the experiment (explants were cultured for three days in DMEM + 20\% FCS to equilibrate to culture conditions before experimentation), suggesting that residual DNA synthesis may account for any labelled cells present. In addition, explants incubated without serum failed to develop any outgrowth or monolayer in contrast to serum containing cultures. These findings are in agreement with several previous studies. Osborn et al., (1989) reported reduced levels of \[^3\text{H}\]-TdR incorporation in bovine cartilage explants cultured with IGF-1 compared to 20\% FCS. Luyten et al (1987) noted that bovine articular cartilage cultured with IGF-1 failed to form a cellular outgrowth at the margins of the explant.

The induction of DNA synthesis in quiescent cells can be resolved into two phases termed competence and progression which may be induced by different stimuli (Pledger et al., 1977). It has previously been suggested that IGF-1 acts as a progression factor in the cell cycle (Pledger et al., 1978). In order for cells to respond to IGF-1 and, therefore, progress through the cycle, a competence stimulus is required. Competence stimuli may be growth factors or a stimulus such as matrix damage, cell attachment or a change in cell shape. It is
possible that chondrocytes cultured with IGF-1 lack the competence stimulus necessary to induce IGF-1 responsiveness and progression through the cell cycle. Neither PG depletion or the loss of pericellular collagen were found to be sufficient to induce competence as indicated by the lack of DNA synthesis in control explants, which deplete themselves with time, or in chondrocyte culture in which the pericellular matrix is removed. Serum contains a mixture of growth factors including IGF-1, but also contains competence factors such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (Pledger et al., 1978; Prins et al., 1982). In the presence of serum both competence and progression stimuli are present and so cell cycle progression is possible. It is to be expected, therefore, that DNA synthesis is elevated in explants cultured with serum when compared to serum-free conditions.

As mentioned above [3 H]-TdR incorporation is not necessarily an indication of cell division. If mitosis does occur, it leads to the formation of two separately labelled nuclei adjacent to each other. This may be termed a labelled couplet. Cartilage has advantages over other tissues in that deep zone chondrocytes possess a distinct pericellular microenvironment surrounded by a fibrous capsule. It is, therefore, relatively straightforward to look for two labelled nuclei residing within the same capsule in the deep zones of the tissue. In control explants, labelled couplets first appeared between five and seven days. After this point the number of labelled couplets rose linearly up to 14 days. Even at the end of the culture period, however, only 3% of labelled cells were present as couplets. A labelled couplet will only be visualised if both nuclei are in the plain of section and, as a consequence, values presented may be lower than the actual value. The data suggested that chondrocyte cell cycle time is at least five days and that the majority of labelled cells had not progressed through mitosis within 14 days. Whether this is an indication of G2 arrest or merely of a long and variable G2 phase is unclear. Whilst culturing the tissue for an extended period may provide the answer, labelling cells with [3 H]-TdR for long periods can cause problems in itself, due to mutations, altered cellular processes, altered cell cycle kinetics or even cell death (Cleaver et al., 1972; Ehmann et al., 1975; Maurer, 1981).

DNA synthesis has previously been shown to be associated with cartilage damage in arthritis (Rorhwell & Bentley, 1973; Dustmann et al., 1974; Hirotani & Ito, 1975), experimentally induced arthritis (Telhag, 1972; Telhag, 1973; Mayor & Moskowitz, 1974; Fengler & Franz, 1983) and enzymically or physically induced cartilage damage (Havdrup & Telhag, 1978; Johnell & Telhag, 1978; Havdrup, 1979; Kunz et al., 1979). The system described in chapters 1 and 2, whereby enzymes have been used to selectively damage cartilage matrix, has been used to investigate any correlation. In chapter 1 we indicated that hyaluronidase treatment induced an increased loss of PG from the tissue as compared to control levels. Collagen loss and architecture was, however, unaffected. No difference was found between the number or location of [3 H]-TdR labelled cells present in control and
hyaluronidase-treated explants. There is no evidence to suggest, therefore, that proteoglycan depletion alone is sufficient to induce re-entry into the cell cycle.

As reported in chapters 1 and 2, collagenase treatment caused an increased loss of collagen from the tissue and also induced changes in the collagen architecture of the matrix. In collagenase-treated explants the number of [H]-TdR labelled cells was significantly greater than in control explants (p < 0.05 for both concentrations at all time points except 20 unit.ml⁻¹ collagenase at 1 day). The increase was almost entirely due to additional labelled cells present within collagenase-induced damaged tissue. Collagenase-treatment caused disruption of the surface cartilage releasing chondrocytes which subsequently attached and flattened to the underlying undamaged tissue. Cells flattened in this way showed high levels of labelling. In addition cartilage damage at the edges of the explant resulted in the formation of clumps or nodules of cells. A high percentage of the chondrocytes within nodules were labelled with [H]-TdR, in particular the flattened cells found as a sheath surrounding the nodule. The onset of DNA synthesis in articular cartilage was, therefore, associated with disruption in matrix architecture and with the ability of chondrocytes released from the surrounding matrix to attach and flatten to a substrate.

The majority of previous studies mentioned above were in vivo and as a result suffer from a complexity not associated with in vitro explant culture. The cartilage in these studies was altered in several ways, including PG depletion, collagen damage, effects due to synovial inflammation and altered patterns of compressive loading. The complexity of such systems makes it difficult to identify the controlling influences on DNA synthesis. The use of cartilage explant cultures in this study negates the effect of other tissues on cartilage responses. By using this system we have determined that several factors may play a role in the control of DNA synthesis in articular cartilage. These factors are, matrix hindrance (in particular hindrance due to collagen architecture), cell shape and attachment and the presence of growth factors. In order to investigate controlling influences further, isolated chondrocytes were cultured as a monolayer or over agarose, in the presence of serum, IGF-1 or in serum-free condition. Serum was found to be a major controlling factor. Chondrocytes cultured in the presence of serum incorporated significantly more [H]-TdR than in serum-free conditions or in the presence of IGF-1 (p < 0.05 for both culture conditions and time points except 4 day agarose suspension culture). When cultured in the absence of serum or in the presence of IGF-1 the majority of cells remained rounded in both culture systems. Serum contains adhesion proteins such as vitronectin and fibronectin and, therefore, when serum is absent cells fail to flatten due to the lack of these proteins. It is unclear whether the reduction in DNA synthesis observed in serum-free cultures was due to the absence of essential serum growth factors (for instance IGF, FGF or PDGF) necessary for cell cycle progression or because the cells failed to attach and flatten. Vetter et al. (1985 & 1986) have reported a slight increase in the proliferation of rounded chondrocytes above the levels
found in medium alone. The levels of proliferation were, however, markedly lower than in medium supplemented with 20% FCS. A proliferative response to IGF-1 is well known in cultured chondrocytes when they are allowed to flatten (Van Wyk et al., 1974; Taylor et al., 1988; Froger-Gaillard et al., 1989) and it is, therefore, possible that if the cells had been allowed to flatten that IGF-1 responsiveness may be achieved. Certainly, the number of IGF-1 receptors is believed to be greater in flattened than rounded chondrocytes (Trippel et al., 1983; Watanabe et al., 1985). Monolayer cultures incorporated significantly more [3H]-TdR than the equivalent agarose suspension cultures, suggesting that the ability of cells to attach and spread on a substrate plays an important controlling role in DNA synthesis. This theory is further supported by the observation that between 4 and 8 days chondrocytes cultured over agarose began to attach and spread. This was coupled with an increase in DNA synthesis during this period. The association between cell attachment and DNA synthesis in chondrocyte cultures is well known and the results presented are in agreement with several previous investigations (Sokoloff et al., 1973; Benya & Shaffer, 1982; Keuttner et al., 1982; Glowacki et al., 1983; Sokoloff, 1985; Aydelotte & Kuettner, 1988; Watt, 1988; Watt & Dudhia, 1988). All these authors have reported that increased proliferation occurs when chondrocytes are allowed to spread compared to rounded chondrocytes. It is possible, therefore, that cell attachment and flattening acts as a competence stimulus, allowing the cells to respond to progression factors and advance through the cell cycle.

The control of DNA synthesis in adult articular chondrocytes is a complex process in which several factors may be of importance. Factors found to stimulate DNA synthesis in this study were, removal or disruption of collagenous matrix from around the cell, conditions which allow the cells to attach and flatten on a substrate and the presence of serum factors. These conditions may act separately or together to induce differing degrees of DNA synthesis and, as such, it is difficult to ascertain the relative importance of these factors in the control of chondrocyte division.
CHAPTER 4.

PROTEOGLYCAN SYNTHESIS IN MATRIX-DEPLETED ARTICULAR CARTILAGE.
Introduction.

The turnover of proteoglycans in articular cartilage involves degradation and loss of existing molecules and also the production of new proteoglycans. In healthy cartilage synthesis is sufficient to replace lost proteoglycans and, therefore, maintain functional integrity.

Proteoglycan synthesis is a highly complex process involving first the translation of PG core protein, the subsequent addition of O- and N-linked oligosaccharides and chondroitin and keratan sulphate side chains and finally the sulphation of such chains (Helting & Roden, 1969a,b; De Luca et al., 1973; Nilsson et al., 1982; Thonar et al., 1983; Carney & Muir, 1988). Once synthesised, proteoglycans must be transported out of the cell and form aggregates if they are not to be lost from the matrix. It is believed that post secretional maturation occurs within the matrix to allow aggregation to occur. This process probably involves the formation of stable disulphide bridges within the hyaluronan-binding region of the proteoglycan core protein (Oegema, 1980, Bayliss et al., 1983, 1984). The complexity of the system means that regulation of synthetic rate and proteoglycan structure could take place at any one of a number of stages.

Whilst in normal tissue, proteoglycans are produced at a rate that balances degradation and loss, certain diseased and damaged states are known where changes occur in the quantity and type of proteoglycan synthesised. In human osteoarthritic cartilage there is an increase in the rate of proteoglycan degradation, which is accompanied by an increase in proteoglycan synthesis (Mankin, 1973; Ryu et al., 1984). Similar findings have been found in experimental models of osteoarthritis both in vivo and in vitro (McDevitt et al., 1981; Sandy et al., 1984). This phenomenon has been explained as a feedback mechanism, by which chondrocytes attempt to maintain proteoglycan levels, which have been reduced in arthritic conditions, by increasing synthesis. This theory is supported by several studies. When embryonic chick rudiments, grown in organ culture, are treated with papain or hyaluronidase so that much of the proteoglycan is lost, the chondrocytes respond by rapid synthesis of new matrix components (Bosmann, 1968; Fitton-Jackson, 1970; Hardingham et al., 1972). When removed from enzyme containing medium, proteoglycan levels rapidly return to normal. In a similar fashion, when chondrocytes are isolated from cartilage and cultured in suspension or as a monolayer, the cells typically re-synthesise a surrounding matrix.

The mechanisms by which chondrocytes detect changes in the surrounding matrix and then respond to them are unclear. Hyaluronan has been suggested as a regulator of proteoglycan synthesis, as it has an inhibitory effect on synthesis in adult and embryonic chondrocytes (Wiebkin & Muir, 1973; Toole, 1973; Solursh et al., 1974). The effect is believed to be regulated by hyaluronan bound to the cell surface, but not in aggregates.
(Wiebkin et al., 1975). The levels of growth factors and metabolites may be altered in damaged and diseased cartilage. There is evidence to suggest that insulin-like growth factors (IGFs) are important regulators of proteoglycan synthesis in vivo and in both isolated chondrocytes and in explant cultures (Daughaday et al., 1972; McQuillan et al., 1986a; Vetter et al., 1985; Luyten et al., 1988). Other growth factors and cytokines, such as transforming growth factor-β (TGF-β) and the interleukins may also act as regulators of proteoglycan synthesis (Morales & Hascall, 1989).

In addition to quantitative changes, qualitative alterations in the type of proteoglycan produced are also known to occur in diseased and damaged tissue. The proteoglycans produced in osteoarthritic cartilage are larger than normal due to substitution of longer chondroitin sulphate chains (Carney et al., 1985a, b). Altered sulphation patterns also occur, including over-sulphated regions of the chondroitin sulphate chains and abnormal terminal sequences (Caterson et al., 1990; Sorrell et al., 1990). It is of interest, therefore, to investigate the regulation of proteoglycan synthesis in cartilage and how it is altered, both quantitatively and qualitatively in experimentally damaged tissue.

The object of the present study is to measure proteoglycan synthesis in adult articular chondrocytes under various in vitro conditions. The incorporation of radioactive sulphate has been used to assess synthesis. This technique is widespread in the study of proteoglycan synthesis as radioactive sulphate is incorporated into the glycosaminoglycan side chains of proteoglycans during synthesis. The bovine explant system has been used extensively in synthesis studies and is believed to show steady-state kinetics during culture (Hascall et al., 1983; Campbell & Handley, 1987; Campbell et al., 1984 a,b, 1989; Bartholomew et al., 1985; McQuillan et al., 1984, 1986a,b,c; Luyten et al., 1988). In order to assess the effect of the matrix on proteoglycan synthesis, explants of adult articular cartilage have been subjected to enzyme treatment as described and reported in chapters 1 and 2. In addition, the effect of serum (which contains several growth factors in varying concentrations) and purified IGF-1 on proteoglycan synthesis in explant and cell culture has been measured. In order to assess the effect of cell shape and attachment on the regulation of proteoglycan synthesis, chondrocytes, isolated from cartilage have been cultured as a monolayer, in suspension over agarose or within Gel-Wells™. In addition to quantitative studies, qualitative changes in proteoglycan synthesis have been assessed using immunolocalisation. The antibodies used recognise normal chondroitin-4- and -6-sulphate and novel epitopes associated with damaged and repair tissue (Caterson et al., 1985, 1987, 1989; Sorrell et al., 1988, 1990; Hardingham et al., 1989).
**Materials and Methods.**

Radioactive sulphate labelling

Explants were set up and cultured as before. At specified times during the culture period explants were transferred to a new 35 mm dish containing 1 ml fresh medium supplemented with 10 μCi.ml⁻¹ S as inorganic sulphate, (³⁵SO₄), (Amersham International plc, Amersham, England). The labelled cultures were returned to the incubator and incubated for a further 24 hours. The length of the labelling period was sufficient to minimise the effect of non-steady state synthesis before equilibration of ³⁵SO₄ within the tissue had occurred (Maroudas & Evans, 1974). At the end of the labelling period the culture medium was removed and stored frozen prior to analysis. The tissue was diced finely and transferred to a cryotube containing 1 ml 4M guanidine hydrochloride made up in 20 mM sodium acetate buffer plus protease inhibitors (1.25 mg.ml⁻¹ N. ethylmaleimide, from Sigma, Poole, England, 1.56 mg.ml⁻¹ benzamidine and 0.5 mg.ml⁻¹ phenyl-methyl-sulphonyl fluoride, both from Aldrich, Gillingham, England). Extraction of proteoglycans was performed for 24 hours at 4°C. The extract was removed and the tissue residue solubilised by digestion in 1 ml papain solution at 60°C for 24 hours.

The effect of serum and Insulin-like growth factor-1 on ³⁵Sulphate incorporation

Explants were set up as previously and cultured in DMEM + 20% (v/v) FCS or in DMEM without added serum or in DMEM supplemented with 50 ng.ml⁻¹ Insulin-like growth factor-1 (IGF-1). At time 0, 2, 8 and 15 days explants were labelled with ³⁵SO₄ as described above. The incorporation rate of ³⁵SO₄ was assessed quantitatively, as described below, from the resulting medium, extract and papain digested samples. The incorporation rate was recorded at the end of the labelling period, i.e. days 3, 9 and 16.

The effect of matrix depletion on ³⁵Sulphate incorporation

Explants were set up and depleted using either hyaluronidase (10 unit.ml⁻¹), or collagenase (20 or 100 unit.ml⁻¹) for 24 hr as previously described and then cultured for a further 13 days. At time 0, 2, 5, 8, 11 and 15 days explants were labelled with ³⁵SO₄ for 24 hr as described above to give incorporation rate at time 3, 6, 9, 12 and 16 days. The incorporation rate of ³⁵SO₄ was assessed quantitatively, as described below, from the resulting medium, extract and papain digested samples.

³⁵Sulphate incorporation in cultures of isolated chondrocytes

Chondrocytes were isolated using the procedure described in chapter 3. Monolayer and agarose suspension cultures were set up as in chapter 3. In addition, a third culture system was employed by culturing cells within Gel-Wells™ (Costar, from Northumbria Biologicals, Cramlingham, England). Gel-Wells™ are made from a gel which permits the diffusion of medium and posses a central cup (volume 200 μl). Cultures were prepared by
placing a single Gel-Well™, cup side up, in a 35 mm tissue culture dish. Isolated chondrocytes were resuspended in medium at a concentration of $1.25 \times 10^6$ ml$^{-1}$ and 200 μl added to the central well. Subsequently, 1 ml fresh medium was added directly to the dish so that it surrounded the Gel-Well™. The cultures were fed by removing the medium from around the Gel-Well™ and replacing it with 1 ml fresh medium.

In order to assess $^{35}$SO$_4$ in monolayer, agarose suspension and Gel-Well™ cultures, 1 ml of medium supplemented with 10 μCi.ml$^{-1}$ $^{35}$SO$_4$ was added to each culture and then incubated for 24 hr. The medium was removed and stored, frozen, prior to analysis. Chondrocytes from monolayer and agarose suspension cultures were harvested as described in chapter 3. Chondrocytes cultured within Gel-Well™ were harvested as follows. The medium from the interior of the well was removed and any cells suspended within it were isolated by centrifugation. Two hundred μl of papain solution was added to the well and incubated at 60°C for 2 hr to remove cells which had attached to the gel. The papain digest, containing detached cells, was removed from the well and added to the cell pellet previously isolated. The volume was made up to 1 ml with papain solution and incubated for a further 16 hr at 60°C.

Total incorporation of $^{35}$SO$_4$ in medium and papain digest samples from monolayer, agarose suspension and Gel-Well™ cultures was assessed using the following method.

**Quantitative assessment of $^{35}$Sulphate incorporation**

In order to determine the total quantity of $^{35}$SO$_4$ incorporated into proteoglycans, it was first necessary to separate bound and unbound isotope. This was achieved by passing medium, extract and papain digest samples down a PD10 column packed with Sephadex G-25 M (Pharmacia Ltd, Milton Keynes, England). The columns were loaded with 0.1 ml sample and then 0.5 ml aliquots of PBS were added and collected, in scintillation vials, as 12, 0.5 ml fractions. Four ml of scintillator 299 (Canberra-Packard, Pangbourne, England) was added to each of the fractions and the total radioactivity present measured using a Triacarb 400 series counter (Packard, Pangbourne, England). A 10 μl aliquot of medium was also counted to determine total counts in the medium. Columns were washed with at least 40 ml PBS before and after use to remove any $^{35}$SO$_4$ still present. Bound $^{35}$SO$_4$ was typically eluted in fractions 5-8 and appeared as a broad peak additional to the free sulphate elution profile. The counts in fractions containing bound sulphate were added to give a value of total counts incorporated. The DNA content of the tissue samples was calculated using the method described in chapter 3.

Using the following assumptions, the incorporation rate of sulphate may be expressed as: mM SO$_4$ incorporated. μg DNA$^{-1}$.hr$^{-1}$.

1. The proportional incorporation of $^{35}$SO$_4$ is the same as cold sulphate.
2. [$^{35}$SO$_4$] is small compared to [cold sulphate], such that the total of sulphate in the medium may be taken as [cold sulphate] = 0.8 mM in Dulbecco’s MEM.
Immunolocalisation of proteoglycan epitopes

In order to ascertain the type of glycosaminoglycan within the tissue and the possible presence of novel epitopes, not found in normal articular cartilage, immunolocalisation was carried out using the primary antibodies described in table 4.1 below.

<table>
<thead>
<tr>
<th>Enzyme Treatment</th>
<th>Epitope Recognised.</th>
<th>Source of immunogen.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3B3 IgM. Chondroitinase ABC</td>
<td>-6-sulphated N-acetylgalactosamine next to an unsaturated hexuronic acid.</td>
<td>Rat chondrosarcoma. Raised in mouse.</td>
</tr>
<tr>
<td>3B3 No enzyme treatment</td>
<td>Native epitope at the non-reducing end of chondroitin sulphate chain.</td>
<td>As above.</td>
</tr>
<tr>
<td>2B6 IgG. Chondroitinase ABC</td>
<td>-4-sulphated N-acetylgalactosamine next to an unsaturated hexuronic acid.</td>
<td>Rat chondrosarcoma. Raised in mouse.</td>
</tr>
<tr>
<td>7D4 IgM. No enzyme treatment</td>
<td>Oversulphated region within chondroitin sulphate chains.</td>
<td>Chick embryo bone. Raised in mouse.</td>
</tr>
</tbody>
</table>

**Table 4.1.** Details of the antibodies used for immunolocalisation of proteoglycan epitopes in control and enzyme-treated bovine articular cartilage. All are monoclonal antibodies. All antibodies were kindly donated by Dr Bruce Caterson, University of North Carolina, Chapel Hill.

Bovine articular cartilage explants were set up, cultured and enzyme treated as before. Directly after enzyme incubation and after a further 6 and 13 days culture, explants were fixed for 2 hr at 4°C in 95% alcohol. Tissue samples were subsequently dehydrated in 100% alcohol (three changes at 4°C) and cleared in xylene (two changes at 4°C). Wax infiltration, prior to embedding, was restricted to 60 min at 60°C to reduce epitope destruction. Wax blocks were stored at 4°C prior to sectioning. Five μm sections were cut using a Spencer 810 microtome and the sections mounted on slides which had previously been de-greased by washing in 70% alcohol. The prepared slides were stored at 4°C.

Immunolocalisation procedure was as follows:

Sections were dewaxed and rehydrated using an alcohol sequence.

- Wash in PBS 2 x 5 min.
- Wash in 1% H₂O₂ in 100% alcohol 10 min.
Wash in PBS

[Incubate with 0.25 unit.ml\(^{-1}\) chondroitinase ABC (Sigma, Poole, England)]

Wash in PBS

Cover with non-immune serum (NIS, 1:20 dilution)

Incubate with 1\(^{st}\) antibody (1:100 dilution in PBS)

Wash in PBS

Incubate with rabbit anti-mouse immunoglobulin.

(used at 0.2 mg.ml\(^{-1}\) in PBS, Sigma, Poole, England)

Wash in PBS

Incubate with peroxidase anti-peroxidase + 1% NIS

(40\(\mu\)g.ml\(^{-1}\) in PBS, Sigma, Poole, England)

Wash in PBS

Cover sections with diamino-benzidine (DAB) solution

(0.5 mg.ml\(^{-1}\) in PBS + 0.05% H\(_2\)O\(_2\), Sigma, Poole), which was filtered before use.

Incubate until colour develops (1-5 min.).

Development of DAB staining was standardised by developing a single section until good staining had appeared and then developing the remaining sections for the same time. Colour development was stopped by immersing sections in distilled water. The sections were dehydrated, cleared in xylene and mounted using Styrolite mounting medium (Raymond A. Lamb, London, England). The sections were viewed and photographed using a Olympus BH2 microscope.

Results

Absolute incorporation rates of sulphate were found to vary from animal to animal and so the results presented in this chapter represent rates from explants removed from both of the metacarpalphalangeal joints from a single animal. Replicate experiments gave a similar overall synthesis pattern over the culture period.

The effect of serum and Insulin-like growth factor-1 on \(^{35}\)Sulphate incorporation

The rate of incorporation of sulphate by chondrocytes from explants incubated for 16 days in DMEM + 20% (v/v) FCS, DMEM (without serum) or DMEM + 50 ng.ml\(^{-1}\) IGF-1 was calculated. Results are presented in Fig. 4.1 (pages 130-131). In addition the percentage of total incorporated \(^{35}\)SO\(_4\) present in the medium, guanidine HCl extract and papain-digested residue was also calculated and presented in Fig. 4.2 (pages 132-133).

In explants cultured in DMEM + 20% (v/v) FCS, (control cultures), the rate of incorporation rose over the first 3 days but then dropped off to below the day 0 value by day 16. The incorporation rates in explants cultured in DMEM + 50 ng.ml\(^{-1}\) IGF-1 showed no
statistically significant difference from control values. Explants incubated without serum incorporated significantly less sulphate than the other culture conditions ($p < 0.05$ at all time points), with the synthesis rate dropping off from day 0.

The proportion of $^{35}\text{SO}_4$ in the medium, guanidine HCl and papain digest from control cultures showed little alteration with time, although there was a slight increase in the proportion of labelled PG present in the medium of older cultures. Certain changes were apparent in the other culture conditions, manifested as an increase in the proportion of PG in the medium and papain digest and a decrease in the guanidine HCl extract of explants cultured in medium without serum. Changes in explants cultured in DMEM + 50 ng.ml$^{-1}$ IGF-1 were less pronounced, but there appeared to be a slight increase in the proportion of labelled PG in the medium fraction.

**The effect of matrix depletion on $^{35}$Sulphate incorporation**

Sulphate incorporation rates in explants cultured in DMEM + 20% FCS, incubated for 24 hr with hyaluronidase or collagenase or without enzyme treatment (control) are presented in Fig. 4.3 (pages 134-137). In addition the percentage of total incorporated $^{35}\text{SO}_4$ present in the medium, guanidine HCl extract and papain-digested residue was also calculated and presented in Fig. 4.4 (pages 138-140).

In control cultures, the synthetic rate increased from day 0-3, but then dropped off to reach a plateau after approximately day 9, which was lower than the day 0 incorporation figure. As such these values are very similar to the equivalent culture conditions presented in Fig. 4.1b. The incorporation rate in hyaluronidase-treated cultures was statistically indistinguishable from control values ($0.05 < p$ at all points except day 9). Similarly, in explants incubated with 20 unit.ml$^{-1}$ collagenase for 24 hr, no difference from control values was detected ($0.05 < p$ at all time points). In contrast, explants incubated with 100 unit.ml$^{-1}$collagenase showed enhanced synthetic rates throughout the culture period ($p < 0.05$ at all time points except day 16).

Alterations in the proportion of labelled PG in the medium, guanidine HCl extract and papain digested residue of enzyme-treated explants, when compared with controls were complex and, therefore the following statements are generalisations. In general, there was an increase in the proportion of labelled PG in the medium of both hyaluronidase and collagenase-treated cultures compared to control values. These differences were most noted soon after enzyme-treatment. Hyaluronidase-treated cultures also appeared to contain a smaller proportion of labelled PG in the guanidine HCl fraction and an increased amount in the papain digest. The papain fraction was often decreased in collagenase-treated cultures, whilst, in general, the guanidine HCl fraction remained unchanged.

$^{35}$Sulphate incorporation in cultures of isolated chondrocytes

Chondrocytes, isolated from full-depth articular cartilage were cultured as a monolayer, in suspension over agarose and in Gel-Wells$^{\text{TM}}$. The morphology of cells in
monolayer and agarose suspension culture has been described in chapter 3. Cells in Gel-Wells™ aggregated to form a ring around the edge of the well. With time a small percentage of cells began to flatten onto the Gel-Well™. The majority of chondrocytes remained rounded and in high density within aggregates.

The rate of incorporation of sulphate by chondrocytes cultured as a monolayer, in suspension over agarose or within Gel-Well™ has been calculated and is presented in Fig. 4.5 (page 141). Monolayer cultures showed an increase in synthesis between day 1 and 4, but the rate dropped off subsequently. Similar alterations with time were detected in both agarose suspension cultures and Gel-Well™ cultures, although the rates were significantly less than in monolayer cultures (p < 0.05 at all time points). No significant difference was detected between incorporation rates in agarose suspension and Gel-Well™ cultures.

**Immunolocalisation of proteoglycan epitopes**

In order to preserve antigenicity, processing and sectioning of cartilage samples was performed at low temperature whenever possible. Normally, however, it is difficult to get cartilage sections to stick to slides without folding unless high temperatures are used. The sections used in this study tended to be folded and often became detached during immunolocalisation causing an unavoidable reduction in quality of the stained sections. In addition, the DAB method used to visualise antibody binding often produced very pale staining. It was difficult, therefore, to photograph the sections, and as a consequence, the quality of some of the photographs presented is poor. In hindsight, and given more time it would be advantageous to investigate the use of enhancement techniques such as silver enhancement to improve the quality of immunolocalisations produced.

Photomicrographs of sections labelled with antibodies have been presented as follows:

- 3B3 (with chondroitinase treatment) Fig. 4.6 (pages 142-143).
- 3B3 (without chondroitinase treatment) Fig. 4.7 (pages 144-145).
- 2B6 (with chondroitinase treatment) Fig. 4.8 (pages 146-147).
- 7D4 (without chondroitinase treatment) Fig. 4.9 (pages 148-149).

In addition the intensity of staining in the surface, deep, disrupted tissue and outgrowth of control and enzyme-treated explants, cultured for up to 16 days, has been assessed and presented in Fig. 4.10 (pages 150-151).

With chondroitinase-treatment 3B3 epitope was found throughout the tissue of control explants. With time there was a progressive reduction in staining, revealing intense pericellular staining. Staining was reduced in both hyaluronidase and collagenase-treated explants directly after enzyme incubation. With time, however, staining returned, with a preferential pericellular location in hyaluronidase-treated tissue and throughout the matrix of collagenase-treated explants.

Without chondroitinase-treatment, 3B3 epitope was absent at day 0 but began to be
present around deep zone cells by day 3. The pattern of distribution was similar in hyaluronidase and collagenase-treated explants.

2B6 epitope was found throughout the matrix of control explants. In a similar fashion to 3B3 (with chondroitinase) there was a progressive reduction in staining with time, revealing intense pericellular staining. Staining was reduced in both hyaluronidase and collagenase-treated explants directly after enzyme incubation. With time, however, staining returned, with a preferential pericellular location in hyaluronidase-treated tissue. Staining was particularly strong in the disrupted tissue and outgrowths from collagenase-treated explants.

7D4 epitope was absent at day 0 but began to be present at the surface of control tissue after day 3. Deep zone tissue stained after 16 days in culture. Staining in hyaluronidase-treated explants followed a similar pattern, whilst staining was stronger in tissue which had been collagenase treated.
Fig. 4.1a. Graph representing the rate of incorporation of SO₄ by chondrocytes, measured by incubating explants in medium containing 10 μCi.ml⁻¹³⁵SO₄. Explants were cultured in medium supplemented with 20% (v/v) FCS, medium alone or medium supplemented with 50 ng.ml⁻¹ IGF-I. Each point represents the mean of four replicates.

- □ DMEM + 20% (v/v) FCS.
- ■ DMEM (without serum).
- ▲ DMEM + 50 ng.ml⁻¹ IGF-I.

For clarity, each graph has been plotted individually in Fig. 4.1b-d. These figures include error bars and an indication of the difference, significant or otherwise between control medium (DMEM + 20% FCS) and other treatments.

Fig. 4.1b. Graph representing the rate of incorporation of SO₄ by chondrocytes from explants cultured in DMEM + 20% FCS. Error bars indicate the mean ± standard deviation of the mean.
Fig. 4.1c. Graph representing the rate of incorporation of $\text{SO}_4$ by chondrocytes from explants cultured in DMEM (without serum). Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control medium (DMEM + 20% FCS) as follows;

- $p \geq 0.05 = \text{NS.}$
- $0.05 > p \geq 0.01 = *$
- $0.01 > p \geq 0.001 = **$
- $0.001 > p = ***$

Fig. 4.1d. Graph representing the rate of incorporation of $\text{SO}_4$ by chondrocytes from explants cultured in DMEM + 50 ng.ml$^{-1}$ IGF-I. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control medium (DMEM + 20% FCS) as follows;

- $p \geq 0.05 = \text{NS.}$
- $0.05 > p \geq 0.01 = *$
- $0.01 > p \geq 0.001 = **$
- $0.001 > p = ***$
Fig. 4.2. Graphs representing the proportion of total incorporated $^{35}$SO$_4$ present in the medium, guanidine HCl extract and papain-digested residue from explants incubated with 10 μCi.ml$^{-1}$ $^{35}$SO$_4$ for 24 hr on day 3 of culture (Fig. 4.2a), day 9 of culture (Fig. 4.2b) and day 16 of culture (Fig. 4.2c). Each column represents the mean value of four replicates. Error bars indicate the mean plus the standard deviation of the mean. Explants were cultured in the following medium:

- DMEM + 20% (v/v) FCS.
- DMEM (without serum).
- DMEM + 50 ng.ml$^{-1}$ IGF-1.

Unpaired student's t-test values indicate differences from explants incubated in DMEM + 20% FCS as follows:

- $p \geq 0.05 = \text{NS.}$
- $0.05 > p \geq 0.01 = \star$
- $0.01 > p \geq 0.001 = \star\star$
- $0.001 > p = \star\star\star$
Fig. 4.2a. Day 3.

Fig. 4.2b. Day 9.

Fig. 4.2c. Day 16.
**Fig. 4.3a.** Graph representing the rate of incorporation of $\text{SO}_4$ by chondrocytes from explants cultured in DMEM + 20% FCS for 2 days, then incubated with enzymes for 24 hours (from Day 2-Day 3) and then cultured for a further 13 days. Each point represents the mean value of four replicates.

- □ — Control.
- ■ — 10 unit.ml$^{-1}$ hyaluronidase.
- ○ — 20 unit.ml$^{-1}$ collagenase.
- ▲ — 100 unit.ml$^{-1}$ collagenase.

For clarity, each graph has been plotted individually in Fig. 4.3b-e. These figures include error bars and an indication of the difference, significant or otherwise between treated and control points.

**Fig. 4.3b.** Graph representing the rate of incorporation of $\text{SO}_4$ by chondrocytes from explants cultured for 16 days in DMEM + 20% FCS (Control). Error bars indicate the mean ± standard deviation of the mean.

**Fig. 4.3c.** Graph representing the rate of incorporation of $\text{SO}_4$ by chondrocytes from explants incubated for 2 days in DMEM + 20% FCS, then for 24 hours in DMEM + 20% FCS + 10 unit.ml$^{-1}$ hyaluronidase and subsequently cultured for a further 13 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's $t$-test values indicate differences from control as follows:

\[ p \geq 0.05 = \text{NS.} \]
\[ 0.05 > p \geq 0.01 = * \]
\[ 0.01 > p \geq 0.001 = ** \]
\[ 0.001 > p = *** \]
Fig. 4.3a.

Fig. 4.3b.

Fig. 4.3c.
**Fig. 4.3d.** Graph representing the rate of incorporation of $SO_4$ by chondrocytes from explants cultured for 2 days in DMEM + 20% FCS, then incubated for 24 hours in DMEM + 20% FCS + 20 unit.ml$^{-1}$ collagenase and subsequently cultured for a further 13 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control as follows;

\[
p \geq 0.05 \quad = \text{NS.}
\]
\[
0.05 > p \geq 0.01 \quad = \ast
\]
\[
0.01 > p \geq 0.001 \quad = \ast\ast
\]
\[
0.001 > p \quad = \ast\ast\ast
\]

**Fig. 4.3e.** Graph representing the rate of incorporation of $SO_4$ by chondrocytes from explants cultured for 2 days in DMEM + 20% FCS, then incubated for 24 hours in DMEM + 20% FCS + 100 unit.ml$^{-1}$ collagenase and subsequently cultured for a further 13 days without enzyme. Error bars indicate the mean ± standard deviation of the mean. Unpaired student's t-test values indicate differences from control as follows;

\[
p \geq 0.05 \quad = \text{NS.}
\]
\[
0.05 > p \geq 0.01 \quad = \ast
\]
\[
0.01 > p \geq 0.001 \quad = \ast\ast
\]
\[
0.001 > p \quad = \ast\ast\ast
\]
Fig. 4.3d.

Fig. 4.3e.
Fig. 4.4. Graphs representing the proportion of total incorporated $^{35}$SO$_4$ present in the medium, guanidine HCl extract and papain-digested residue from explants incubated with 10 $\mu$Ci.ml$^{-1}$ $^{35}$SO$_4$ for 24 hr on day 3 of culture (Fig. 4.4a), day 6 of culture (Fig. 4.4b), day 9 of culture (Fig. 4.4c), day 12 of culture (Fig. 4.4d) and day 16 of culture (Fig. 4.4e). Each column represents the mean value of four replicates. Error bars indicate the mean plus the standard deviation of the mean. Explants were cultured in DMEM + 20% FCS for 2 days, then incubated with enzymes for 24 hours (from Day 2-Day 3) and then cultured for a further 13 days. Control explants were cultured in DMEM + 20% FCS for 16 days.

- Control.
- 10 unit.ml$^{-1}$ hyaluronidase.
- 20 unit.ml$^{-1}$ collagenase.
- 100 unit.ml$^{-1}$ collagenase.

Unpaired student's t-test values indicate differences from control explants, incubated in DMEM + 20% FCS as follows:

- $p \geq 0.05$ = NS.
- $0.05 > p \geq 0.01$ = *
- $0.01 > p \geq 0.001$ = **
- $0.001 > p$ = ***
Fig. 4.4d. Day 12.

Fig. 4.4e. Day 16.
Fig. 4.5a. Graph representing the rate of incorporation of SO₄ by chondrocytes, isolated from full depth cartilage and cultured in suspension over agarose, as a monolayer or in Gel-Wells.

- □ - Cultured in suspension over agarose.
- ■ - Cultured as a monolayer.
- ♦ - Cultured in Gel-Wells.

Each point represents the mean value of four replicates. Error bars indicate the mean ± the standard deviation of the mean.

Fig. 4.5b. Day 1.
Fig. 4.5c. Day 4.
Fig. 4.5d. Day 8.

Fig. 4.5b-d. One way, three group analysis of variance tests showed significant differences between treatment groups at all time points (p < 0.05, full data not shown). Newman-Keuls multiple comparison tests have been used to determine differences between treatment groups (Fig. 4.5b-d). In all cases treatment means have been ranked in ascending order using the following abbreviations:

- M Chondrocytes cultured as a monolayer.
- A Chondrocytes cultured in suspension over agarose.
- G Chondrocytes cultured in Gel-Wells.

NS indicates that the difference between means was not significant at the 5% level, whilst * indicates that the difference was significant at the 5% level.
Fig. 4.6. Immunolocalisation of 3B3 antibody, visualised by DAB staining using chondroitinase-treated sections of bovine articular cartilage. The antibody recognises a -6-sulphated N-acetyl galactosamine next to an unsaturated hexuronic acid.

Fig. 4.6a. Control section, incubated with non-immune rabbit serum in place of the primary antibody. Staining is absent throughout the tissue. Magnification x 40.

Fig. 4.6b. Cartilage fixed immediately after excision from the joint (day 0). Staining is intense throughout the matrix. Magnification x 40.

Fig. 4.6c. Cartilage explant cultured for 2 days after removal from the joint. Staining is intense throughout the tissue. Magnification x 40.

Fig. 4.6d. Control tissue after 16 days in culture. Overall staining is reduced and is primarily in a pericellular location, directly around the chondrocytes. Some in terterritorial matrix staining is, however, apparent. Magnification x 40.

Fig. 4.6e. Tissue cultured for 2 days, then incubated for 24 hr with 10 unit.ml⁻¹ hyaluronidase and fixed directly afterwards. A loss of staining throughout the tissue is apparent. Magnification x 40.

Fig. 4.6f. Tissue cultured for 2 days, then incubated for 24 hr with 10 unit.ml⁻¹ hyaluronidase and cultured for a further 13 days prior to fixation. Some staining is present, mainly pericellularly and in the surface zone interterritorial matrix. Magnification x 100.

Fig. 4.6g. Tissue cultured for 2 days, then incubated for 24 hr with 100 unit.ml⁻¹ collagenase and fixed directly afterwards. Staining is much weaker than in the equivalent control section (Fig. 4.6c) but is still detectable throughout, including in disrupted areas. Magnification x 100.

Fig. 4.6h. Tissue cultured for 2 days, then incubated for 24 hr with 100 unit.ml⁻¹ collagenase and cultured for a further 13 days prior to fixation. Staining is intense throughout the tissue. Whilst the strongest staining is within the main body of the tissue, cellular outgrowths also show staining. Staining is not restricted to pericellular locations. Magnification x 100.
Fig. 4.7. Immunolocalisation of 3B3 antibody, visualised by DAB staining using sections of bovine articular cartilage (without chondroitinase-treatment). The antibody recognises a chondroitin-6-sulphate epitope which has a glucuronic acid terminal sequence.

**Fig. 4.7a.** Control section, incubated with non-immune rabbit serum in place of the primary antibody. Staining is absent throughout the tissue.
Magnification x 200.

**Fig. 4.7b.** Cartilage fixed immediately after excision from the joint (day 0). Staining is absent throughout the matrix.
Magnification x 200.

**Fig. 4.7c.** Cartilage explant cultured for 2 days after removal from the joint. Staining is present in a pericellular location around some cells near to the deep cut edge of the explant.
Magnification x 200.

**Fig. 4.7d.** Control tissue after 16 days in culture. A greater number of cells were found to be positive than after 2 days (Fig. 4.7c). Staining remained exclusively pericellular.
Magnification x 200.

**Fig. 4.7e.** Tissue cultured for 2 days, then incubated for 24 hr with 10 unit.ml⁻¹ hyaluronidase and fixed directly afterwards. Occasional cells showed pericellular staining near the deep cut edge of the explant.
Magnification x 200.

**Fig. 4.7f.** Tissue cultured for 2 days, then incubated for 24 hr with 10 unit.ml⁻¹ hyaluronidase and cultured for a further 13 days prior to fixation. Occasional cells showed pericellular staining in the deep zones of the tissue.
Magnification x 200.

**Fig. 4.7g.** Tissue cultured for 2 days, then incubated for 24 hr with 100 unit.ml⁻¹ collagenase and fixed directly afterwards. Occasional cells showed pericellular staining in the deep zones of the tissue.
Magnification x 200.

**Fig. 4.7h.** Tissue cultured for 2 days, then incubated for 24 hr with 100 unit.ml⁻¹ collagenase and cultured for a further 13 days prior to fixation. A greater number of cells were found to be positive than directly after the enzyme-treatment (Fig. 4.7g). Some staining was apparent beyond the pericellular location.
Magnification x 200.
**Fig. 4.8.** Immunolocalisation of 2B6 antibody, visualised by DAB staining using chondroitinase-treated sections of bovine articular cartilage. The antibody recognises a 4-sulphated N-acetyl galactosamine next to an unsaturated hexuronic acid.

**Fig. 4.8a.** Control section, incubated with non-immune rabbit serum in place of the primary antibody. Staining is absent throughout the tissue.

Magnification x 40.

**Fig. 4.8b.** Cartilage fixed immediately after excision from the joint (day 0). Staining is intense throughout the matrix.

Magnification x 40.

**Fig. 4.8c.** Cartilage explant cultured for 2 days after removal from the joint. Staining is intense throughout the tissue.

Magnification x 40.

**Fig. 4.8d.** Control tissue after 16 days in culture. Overall staining is reduced and appears to be patchy within the matrix. There is some evidence of pericellular staining.

Magnification x 40.

**Fig. 4.8e.** Tissue cultured for 2 days, then incubated for 24 hr with 10 unit.ml⁻¹ hyaluronidase and fixed directly afterwards. Staining is less intense than in the equivalent control section (Fig. 4.8c). There is a tendency for staining to be located in the pericellular environment.

Magnification x 40.

**Fig. 4.8f.** Tissue cultured for 2 days, then incubated for 24 hr with 10 unit.ml⁻¹ hyaluronidase and cultured for a further 13 days prior to fixation. Staining is patchy throughout the matrix. Pericellular staining is apparent.

Magnification x 40.

**Fig. 4.8g.** Tissue cultured for 2 days, then incubated for 24 hr with 100 unit.ml⁻¹ collagenase and fixed directly afterwards. Staining is much weaker than in the equivalent control section (Fig. 4.8c) but is still detectable throughout, including in disrupted areas.

Magnification x 40.

**Fig. 4.8h.** Tissue cultured for 2 days, then incubated for 24 hr with 100 unit.ml⁻¹ collagenase and cultured for a further 13 days prior to fixation. Staining is intense throughout the matrix, including disrupted tissue and outgrowths. Staining is not restricted to pericellular locations.

Magnification x 40.
Fig. 4.9. Immunolocalisation of 7D4 epitope, visualised by DAB staining using sections of bovine articular cartilage (without chondroitinase-treatment). The antibody recognises over-sulphated regions within chondroitin sulphate chains.

Fig. 4.9a. Control section, incubated with non-immune rabbit serum in place of the primary antibody. Staining is absent throughout the tissue.
Magnification x 40.

Fig. 4.9b. Cartilage fixed immediately after excision from the joint (day 0). Staining is absent throughout the matrix.
Magnification x 40.

Fig. 4.9c. Cartilage explant cultured for 2 days after removal from the joint. Staining is present in a pericellular location around the majority of cells near to the surface of the tissue.
Magnification x 40.

Fig. 4.9d. Control tissue after 16 days in culture. A greater number of cells were found to be positive than after 2 days (Fig. 4.9c). Some staining was present in the interterritorial matrix in addition to the pericellular matrix.
Magnification x 40.

Fig. 4.9e. Tissue cultured for 2 days, then incubated for 24 hr with 10 unit.ml⁻¹ hyaluronidase and fixed directly afterwards. There is little staining anywhere in the tissue.
Magnification x 40.

Fig. 4.9f. Tissue cultured for 2 days, then incubated for 24 hr with 10 unit.ml⁻¹ hyaluronidase and cultured for a further 13 days prior to fixation. Pericellular staining is present around cells near the surface and ends of the explant.
Magnification x 40.

Fig. 4.9g. Tissue cultured for 2 days, then incubated for 24 hr with 100 unit.ml⁻¹ collagenase and fixed directly afterwards. Staining is present in the disrupted tissue at the surface of the explant and, occasionally around cells in the deep zones.
Magnification x 40.

Fig. 4.9h. Tissue cultured for 2 days, then incubated for 24 hr with 100 unit.ml⁻¹ collagenase and cultured for a further 13 days prior to fixation. Staining is present throughout the tissue, but is most intense in the surface zones. Disrupted tissue and outgrowths stained less intensely than non-disrupted areas.
Magnification x 40.
Fig. 4.10a. 3B3 with chondroitinase treatment.

Fig. 4.10. Tables indicating the intensity of DAB staining from immunolocalisations prepared from tissue incubated with 3B3 after chondroitinase treatment (Fig. 4.10a) or without chondroitinase treatment (Fig. 4.10b). Intensity has been assessed using the following sequence, (-, ±, +, ++, +++), to indicate intensity of staining varying from no staining (-) to intense staining (+++). There is also an indication of the location of staining within the tissue. In the surface and deep zones the first symbol represents staining in the interterritorial matrix, whilst the symbol in brackets represent pericellular staining. Control and hyaluronidase-treated tissue did not contain disrupted tissue or outgrowth and these figures are not applicable (NA).
Fig. 4.10. Tables indicating the intensity of DAB staining from immunolocalisations prepared from tissue incubated with 2B6 after chondroitinase treatment (Fig. 4.10c) or with 7D4 (Fig. 4.10d). Intensity has been assessed using the following sequence, (−, ±, +, ++, +++), to indicate intensity of staining varying from no staining (−) to intense staining (+++). There is also an indication of the location of staining within the tissue. In the surface and deep zones the first symbol represents staining in the interterritorial matrix, whilst the symbol in brackets represent pericellular staining. Control and hyaluronidase-treated tissue did not contain disrupted tissue or outgrowth and these figures are not applicable (NA).
Discussion.

The object of this chapter was to investigate the rate and type of proteoglycans (PG) synthesised by articular cartilage explants in culture and how it is affected by different culture conditions and by enzymic depletion as characterised in chapters 1 and 2. The rate of PG synthesis was assessed by measuring sulphate incorporation by chondrocytes using radioactive sulphate. The vast majority of sulphate incorporated by chondrocytes is used in the sulphation of PG glycosaminoglycans. The different stages of PG synthesis are believed to be coordinately regulated and, therefore, sulphate incorporation may be used to estimate PG synthesis (Telser et al., 1965; Cole & Lowther, 1969).

Cartilage explants cultured in medium supplemented with 20% (v/v) FCS showed specific alterations in PG synthesis throughout the culture period. During the first three days of culture there was an increase in the rate of synthesis. The absolute increase varied from sample to sample, but was usually approximately 50%. Beyond day 9, a steady drop in synthetic rate was noted up to day 16. The day 16 rate was typically lower than the rate of incorporation directly after removal from the joint. In some cases there appeared to be a plateau of incorporation after day 10, whilst in others the rate continued to fall until the end of the culture period. It was rare for the rate of incorporation to remain constant for any length of time.

These results appear initially to be at odds with previous studies which have suggested that after an initial rise in PG synthesis, during the first few days in culture, synthetic rates remain constant for up to 3 weeks when cultured with 20% FCS (Hascall et al., 1983; Handley et al., 1986; McQuillan et al., 1986a,b; Luyten et al., 1988). The previous studies mentioned measured PG synthesis on the basis of cartilage wet weight, whilst the present study used DNA content as a baseline. As PG synthesis is a cellular phenomenon, it seems sensible to present results on a cellular basis. Indeed, Luyten et al. (1988) have shown that over 3 weeks in culture the DNA content of bovine cartilage explants cultured in medium containing 20% FCS increases by approximately 100% due to the formation of cellular outgrowths. By the end of the culture period, PG synthesis is therefore occurring in twice the number of cells and so the rate per cell is halved. If the previous studies had used the same method for calculating their results a similar reduction in the rate of synthesis per mg DNA would be seen. As PG synthesis is a cellular phenomenon, it seems sensible to present results on a cellular basis.

All studies agree, however, on an initial rise in synthetic rate during the first few days in culture. This is presumably a response to the alterations in environmental factors associated with a change from in vivo to in vitro conditions. Tissue culture medium containing 20% FCS has a much higher concentration of growth factors than synovial fluid, which is primarily involved in the nutrition of articular cartilage in vivo. In addition,
diffusion of growth factors and metabolites into the cartilage is considerably easier in vitro due to the increased surface area of the cartilage. PG synthesis was stimulated by approximately 50% in our studies, whilst Hascall et al. (1983) measured an increase of 300% when transferred to culture conditions. There were slight differences in the culture method used which may explain this anomaly. The tissue used in the present study was, essentially intact, whilst Hascall et al., used finely minced tissue. Growth factor diffusion is likely to be easier in minced tissue and, therefore, the levels in the centre of the cartilage may have been higher than in the present study, inducing a greater stimulation of PG synthesis. The initial rise in PG synthesis has also been interpreted as a response of the tissue to the loss of PG from the matrix which is often associated with culturing (Lane & Brighton, 1974; Benya & Nimni, 1979). This would appear to be unlikely in this case as we have demonstrated that PG depletion occurs throughout the culture period and should, therefore, induce a progressive increase in PG synthesis. No such increase has been demonstrated.

The proportion of newly-synthesised PG in the medium, guanidine HCl extract and papain-digested residue from control cultures on day 1 showed that approximately 5% was lost into the medium, 70% could be extracted and 25% was non-extractable. With time the proportion of PG lost into the medium increased to reach 15% on day 16. We have demonstrated in chapters 1 and 2 that there is a progressive reduction in the PG content of the tissue with time. As the ability of PG monomers to diffuse out of the matrix is highly dependent on the levels of PG within the matrix (Maroudas, 1976), it is possible that it is easier for newly-synthesised PGs to diffuse out of the tissue before stable aggregates have formed on day 16 than on day 1.

PG synthesis in cartilage explants was found to be highly dependent on the presence of serum. In serum-free conditions, PG synthesis was significantly less than in serum-containing medium. The reduction in PG synthesis is probably due to the lack of serum growth factors, some of which are known to be regulators of PG metabolism. There was also an alteration in the proportion of newly-synthesised PG in each fraction. The amount of PG released into the medium was elevated, as was the proportion of non-extractible PG. The guanidine HCl fraction was, consequently, reduced. As mentioned in chapter 1, serum contains inhibitors to the enzymes thought to be primarily responsible for PG breakdown and loss. In the absence of serum, increased proteolytic activity is likely to occur. The reason for an increased non-extractible fraction in serum-free conditions is unclear, although it is possible that due to the overall reduction in synthesis absolute levels of non-extractible PG are unchanged.

Serum contains a variety of factors which are believed to regulate PG synthesis. On the basis of in vitro, clinical and in vivo evidence, it has been suggested that IGF-1 is the primary growth factor responsible for the regulation of PG metabolism in adult articular cartilage (Herington et al., 1983). Articular cartilage, cultured in medium supplemented
with 50 ng.ml\(^{-1}\) IGF-1 showed synthesis rates which were statistically indistinguishable from tissue cultured with 20% FCS. There are several previous reports that IGF-1 can be used as a replacement for serum in maintaining synthesis levels in articular cartilage (Handley \textit{et al.}, 1986; McQuillan \textit{et al.}, 1986a; Luyten \textit{et al.}, 1988). There were slight changes in the proportion of newly-synthesised PG in each fractions. Alterations from values obtained in serum-containing cultures were not consistent and, therefore, it was not possible to draw any conclusions. The use of IGF-1 has advantages over serum in that it is more controllable, since serum shows variation in the levels of growth factors from batch to batch. In addition, cellular outgrowths are not formed in explants cultured with IGF-1 and so matrix architecture is preserved in a more natural state than in serum-containing cultures.

Whilst recognising the advantages of IGF-1 over serum, the work presented in previous chapters has primarily utilised medium supplemented with serum and in the interest of continuity all further work in this chapter also uses serum.

The effect of matrix depletion of PG synthesis in articular cartilage has been assessed using the system characterised in chapters 1 and 2. The rate of synthesis in hyaluronidase-treated cultures was indistinguishable from control explants. This is somewhat suprising as it has previously been reported that enzymatically-induced PG depletion is sufficient to cause an increase in synthesis. The literature is somewhat confused on the issue. Elevated synthesis levels have been reported in papain and hyaluronidase-depleted embryonic cartilage and \textit{in vivo}, following intrarticular papain injections (Bosman \textit{et al.}, 1972; Milroy & Poole, 1974; Bryant \textit{et al.}, 1958). Bartholomew \textit{et al.}, (1985), however, reported an inhibition of PG synthesis in bovine articular cartilage treated with trypsin. Other studies are somewhat vague about the amount of PG synthesis after enzyme-induced depletion (Verbruggen \textit{et al.}, 1985a,b). It must be recognised that hyaluronidase-treatment only caused 30% depletion of PG (chapter 1), whilst previous studies induced 75%-90% depletion. It is possible that the levels of PG depletion induced in this study were insufficient to elicit a response.

Changes were apparent in the proportion of newly-synthesised PG in the medium, guanidine HCl and papain-digested residue of hyaluronidase-treated cartilage as compared to controls. Directly after enzyme incubation there was an increase in the medium fraction, presumably caused by hyaluronidase-induced disruption of PG aggregates. From day 6 onwards there was little evidence of a consistent change in the medium fraction. Surprisingly, the papain fraction was increased throughout the culture period. There is no clear explanation for this phenomenon. It may be that a large proportion of newly-synthesised PGs are retained pericellularly, possibly being associated with newly-synthesised hyaluronan. There is little evidence for this and further experiments would be necessary before a less speculative explanation is proposed.

In explants treated with 100unit.ml\(^{-1}\) collagenase for 24 hr, the PG synthesis rate was
significantly greater than in control tissue during enzyme treatment. PG synthesis remained elevated until day 16 when control levels were restored. The lower concentration of collagenase (20 unit.ml⁻¹) failed to stimulate synthesis. Synthesis levels in collagenase-treated cultures (100 unit.ml⁻¹) were typically between 50 and 100% higher than in control tissue. It is not clear whether elevated synthesis is due to raised levels in the chondrocytes within the main body of the tissue or within disrupted and outgrowth tissue. Directly after enzyme-treatment it is likely that increased metabolism occurs within the main body of the tissue as outgrowths had not formed at this stage. Later during the culture period, however, cellular outgrowths formed at the deep cut edge of the explant which stained intensely with Safranin-O, indicating high PG content produced by de novo PG synthesis. Nodules, formed from disrupted surface tissue, failed to stain with Safranin-O. This may indicate that PG synthesis in these cells is occurring at a low level, or that PGs formed fail to be retained within the matrix. Without further experimentation it is impossible to ascertain the primary location of increased synthesis within collagenase-treated tissue. It is possible, however, to speculate about the regulatory mechanism, which induces enhanced synthesis. The disruption and loss of matrix from around cells may induce a feedback mechanism such that synthetic levels are elevated. Of particular importance may be the pericellular matrix surrounding chondrocytes. We have previously demonstrated in chapter 2 that alterations in the pericellular environment do occur and could lead to a cellular response due to decreased matrix levels at the cell surface. Certainly, the amount of non-extractible PG, the majority of which is believed to be pericellular, is reduced in collagenase-treated tissue. In addition collagenase-disrupted tissue will be more permeable than control tissue. This may lead to elevated levels of growth factors and metabolites within the tissue.

Alterations in the compartmentalisation of newly-synthesised PG between the medium, guanidine HCl and papain-digested residue were noted in collagenase-treated cultures. Throughout the culture period there was an increase in the proportion of PG found in the medium and a reduction in the non-extractible papain fraction. Due to their permanence, it may be assumed that these changes are caused by irreversible changes in the tissue. It is to be expected that a large proportion of newly synthesised PG produced in cells from disrupted areas or from the outgrowth will be lost into the medium, due to the reduced ability for PGs to be incorporated and held within the matrix. Changes within the deep zones of the tissue may increase the permeability of the tissue and thus facilitate the loss of PG. Non-extractible PGs are believed to be associated with collagen or trapped in a pericellular location. Both the pericellular matrix and collagen-PG interactions are disrupted by collagenase resulting in a decreased proportion of PG which cannot be extracted with guanidine HCl.

The results from experiments involving hyaluronidase-treatment of cartilage indicate that PG depletion alone is insufficient to induce increased synthesis. Collagenase-treatment, which caused both PG depletion and collagen architecture disruption, was found to
induce both quantitative and qualitative changes in PG synthesis. It is proposed, therefore, that the collagen architecture of cartilage is more important in vitro in regulating PG synthesis than PG content alone.

In order to investigate the role of cell shape on PG synthesis, cells were isolated from full-depth articular cartilage and cultured as a monolayer, in suspension over agarose or within Gel-Wells™. Monolayer cultures were found to incorporate significantly more sulphate than either agarose suspension or Gel-Well™ cultures. Synthetic rates in agarose suspension and Gel-Well™ cultures were statistically indistinguishable. In all cases synthesis was greatest on day 4 than on both days 1 and 8. The data presented was from a single experiment only. Unfortunately, due to time restrictions it was not possible to repeat the experiment and, therefore, conclusions must be drawn with caution. In the only previous published experiments comparing synthesis in similar culture systems (using human chondrocytes), the incorporation rate in agarose suspension cultures was found to be higher than in monolayer culture (Archer et al., 1990). These findings appear to be at odds with the present results, but other experiments from the same laboratory have indicated increased synthesis in monolayer cultures (Archer, personal communication). It has been suggested that cell shape can regulate PG synthesis, in that rounded cells show greater synthetic rates than flattened ones (Newman & Watt, 1988; Watt & Dudhia, 1989; Archer et al., 1990). The results presented do not support this theory. In both agarose suspension and Gel-Well™ culture, chondrocytes were maintained in high density aggregates. It is possible that cells rapidly resynthesise a surrounding matrix which may act to inhibit further PG synthesis. In monolayer cultures, the majority of newly-synthesised PG was lost into the matrix. It may be assumed, therefore, that monolayer chondrocytes possess less pericellular matrix than in agarose and Gel-Well™ cultures. Feedback inhibition of PG synthesis may not occur in monolayer cultures. Cell density appears to be playing a greater regulatory role than cell shape using the culture systems described.

Immunolocalisation was performed to determine the location and extent of chondroitin-4- and -6-sulphate throughout the matrix and to determine the presence or absence of novel chondroitin sulphate epitopes, normally associated with embryonic and osteoarthritic cartilage. Using the antibodies 3B3 (with chondroitinase-treatment) and 2B6 to localise chondroitin-6- and -4-sulphate respectively (Caterson et al., 1985), both epitopes were found throughout the matrix of freshly dissected cartilage. With time in culture there was a gradual reduction in staining for both epitopes, in accordance with the loss of Safranin-O staining reported previously. Toward the end of the culture period, both epitopes were localised to the pericellular environment of chondrocytes, suggesting de novo synthesis of the molecules. Whilst staining was appreciably weaker in hyaluronidase-treated cultures directly after enzyme treatment, by day 16 staining patterns in control and hyaluronidase-treated sections were essentially the same for both epitopes. In contrast, whilst staining was also reduced in collagenase-treated explant directly after enzyme
treatment, by day 16 staining was strong throughout the matrix. This may be caused partly by the overall increase in PG synthesis found in collagenase-treated cultures, but also because of the inability of PG to be retained pericellularly in explants in which the collagen has been disrupted.

Tissue fixed immediately after dissection did not stain with either 3B3 (without chondroitinase-treatment) or with 7D4. This was expected as these epitopes are not believed to be present in normal adult articular cartilage (Caterson et al., 1990). Suprisingly, however, staining was present in cultured cartilage from day 3 onwards. The 3B3 epitope was primarily located around deep zone chondrocytes, especially near to the cut edge of the tissue. The 7D4 epitope, in contrast was located in the surface tissue. Staining was primarily pericellular, suggesting de novo synthesis of the epitopes rather than modification of existing PG. In both cases staining was enhanced in collagenase-treated tissue and was less pericellular in location. The native 3B3 epitope is exclusively located at the non-reducing termini of chondroitin sulphate chains and its expression probably indicates an alteration in the chondroitin sulphate chain termination process (Caterson et al., 1990). 7D4 is believed to recognise oversulphated regions within chondroitin sulphate chains (Caterson et al., 1990). Expression of this epitope is probably an indication of altered sulphation during chondroitin sulphate synthesis. Both of these epitopes have been detected in experimentally-induced osteoarthritis and it appears that explant culture conditions and collagenase treatment can also induce expression (Hardingham et al., 1989; Caterson et al., 1989, 1990). The relevance of 3B3 and 7D4 epitopes in cartilage is not understood.
GENERAL DISCUSSION
General Discussion.

The object of the present study was to investigate the effect of culture and matrix depletion on the morphology, ultrastructure and metabolism of adult articular cartilage in explant culture. Control explants were cultured for up to two weeks and any changes during this period were assessed. Matrix depletion was achieved by incubating the cartilage for 24 hrs in medium containing either *Streptomyces* hyaluronidase or *Clostridium* collagenase and subsequently culturing the tissue for a further two weeks in medium without enzymes to investigate reparative processes. Both enzymes were of high purity and are known to be highly specific for the substrates, hyaluronan and collagen respectively. In order to assess, quantitatively, the effect of these enzymes on the release of matrix components from cartilage explants, biochemical assays for proteoglycan (PG), hyaluronan and collagen were utilised to determine the amount of component present in the medium and tissue of explant cultures. Morphology and ultrastructure was investigated using light microscopy and both transmission and scanning electron microscopy. DNA synthesis was investigated using tritiated thymidine autoradiography, which allows the number and location of thymidine-labelled cells to be assessed. PG synthesis was also investigated in control and enzyme-treated cartilage, both quantitatively using 35S-incorporation and qualitatively, using immunolocalisation to detect the presence of glycosaminoglycan epitopes not normally present in adult articular cartilage.

Previous studies using explants of bovine articular cartilage have suggested that the levels of matrix components and the rate of synthesis in culture remains constant (Hascall *et al.*, 1983). In our study control explants, cultured without enzymes, exhibited a steady release of PG into the medium throughout the culture period at a rate of about 9% of the total tissue PG per day, such that after 9 days approximately 60% of the total PG was present in the medium. Hyaluronan was also lost from the tissue at a fairly constant rate, but virtually no collagen loss was detected. The matrix was, therefore, slowly becoming depleted of PG, a finding confirmed by a reduction in Safranin-O staining during culture. This casts a doubt on the usefulness of the bovine explant culture system utilised in this study as it failed to maintain constant levels of matrix components throughout the culture period. Similarly, during culture, there was a steady reduction in the rate of PG synthesis, whether explants were cultured in medium supplemented with 20% FCS or 50 ng.ml⁻¹ IGF-1. Anabolic metabolism was, therefore, insufficient to balance PG loss due to catabolic metabolism. The system may be said to be dynamic rather than steady-state. In a dynamic system, investigating the effect of selective depletion or of growth factors on articular cartilage becomes increasingly complex. It would be useful, therefore, to develop a culture system which maintains both the structural integrity and the metabolic balance between anabolism and catabolism such that the levels of matrix components remain constant. *In vivo* cartilage is maintained under load and it is believed that compression is important in the control of metabolism. Cartilage explants in culture are not placed under load and it is possible that
dynamic loading may help to maintain synthetic rates and matrix levels. A study on the
effect of loading on cultured explants would be useful. A change in the type of glycosam-
inglycan synthesised by chondrocytes in explant culture was detected, indicating the
presence of abnormal terminal sequences on chondroitin sulphate chains in the deep zones
and of over-sulphated regions of chondroitin sulphate near to the surface. These epitopes
are normally associated with developing and arthritic tissue. The relevance of these findings
is unclear.

DNA synthesis is believed to be absent in normal adult articular cartilage but was
found to occur at low levels within control explants incubated in medium supplemented
with 20% FCS. In addition cellular outgrowths developed at the edges of control tissue in
serum-containing medium. DNA synthesis within the tissue and the formation of out-
growths were absent, however, when explants were cultured in medium without serum, but
containing IGF-1. In the interest of developing an explant system which most closely
mimics the in vivo state, it would be preferable to use IGF-1 as a medium supplement instead
of serum.

During hyaluronidase incubation an increased proportion of PG was lost from the
tissue when compared to control levels (30% compared to 9%). Similarly, there was an
increase in the loss of hyaluronan. The loss of collagen was, however, indistinguishable
from control tissue. Using Safranin-O staining, the majority of PG depletion in hy-
aluronidase-treated explants appeared to be from the surface zones. It is proposed that this
phenomenon may be due to the lower concentration and smaller size of PGs in the surface
layers which would allow much easier access of the enzyme to hyaluronan substrate than
would occur deeper in the tissue. The diffusion of large molecules through cartilage is
highly dependent on the PG concentration and, therefore, PG monomers would be able to
diffuse out of the tissue more easily in the surface, rather than the deep zones. The
collagenous architecture of hyaluronidase-treated explants was indistinguishable from
control tissue. Hyaluronidase treatment did not produce an increase in the number or
location of cells which had undergone DNA replication, suggesting that PG depletion alone
does not exert a controlling influence on chondrocyte division. Similarly, the rate of PG
synthesis in hyaluronidase-treated cartilage was identical to that in control explants. It has
previously been suggested that chondrocytes in articular cartilage are capable of responding
to a reduction in the PG concentration within the matrix by increasing their synthetic rate
(Hardingham et al., 1972). No evidence to support this theory has been found in this study
in either control or hyaluronidase-treated cartilage.

In collagenase-treated explants there was an increased loss of PG from the tissue,
which reached approximately 40% during the 24 hr incubation with 100 unit.ml−1 col-
lagenase. There was also an increased loss of hyaluronan during collagenase treatment.
Whilst the levels of collagen within the tissue remained constant in control and hy-
aluronidase-treated explants, collagenase treatment induced a significant loss of collagen (measured as hydroxyproline) from the tissue. A collagenase concentration of 20 unit. ml\(^{-1}\) induced a 30% loss of collagen from the tissue, whilst 100 unit.ml\(^{-1}\) produced a 50% loss. Histological examination showed that the greatest tissue disruption in collagenase-treated tissue occurred near to the surface. The enzyme may have greater access to the substrate in the surface zones, due to matrix organisation and PG concentration, and thus cause greater disruption. Using transmission electron microscopy, collagenase appeared to induce a reduction in the thickness of collagen fibrils, presumably due to disruption of interfibrillar crosslinks, prior to complete destruction of fibrillar organisation. The fact that surface zone collagen fibrils are, naturally, thinner than those deeper in the tissue may also explain why disruption is greatest at the surface. Scanning electron microscopic studies revealed disruption of the three-dimensional organisation of collagen matrix, suggesting that collagenase also affects cross-linking between fibrils as well as within fibrils. In the disrupted surface regions, cells released from the matrix had a tendency to form highly cellular nodules. In addition, with time, cellular outgrowths formed at the deep edge of the explant. The outgrowths formed in collagenase-treated tissue were morphologically distinct from those found in control and hyaluronidase-treated explants in that the cells were rounded and surrounded by matrix which stained intensely with Safranin-O. Cellular differences were also apparent, including the appearance of intermediate filaments within chondrocytes and the formation of long cellular processes at the cell surface.

A greater number of cells in collagenase-treated explants were found to have undergone DNA replication, as detected by tritiated thymidine labelling, when compared to control tissue. The effect was dose dependent, in that DNA synthesis was greater in explants treated with 100 unit.ml\(^{-1}\) collagenase than with 20 unit.ml\(^{-1}\). There was a correlation between the extent of tissue disruption and the number of labelled cells present. DNA synthesis was particularly great in areas where cells had become flattened. Whether the major regulating factor is the disruption and loss of collagenous matrix from around the cell or the ability of the cell to flatten is unclear. Certainly, when chondrocytes are removed from their matrix and cultured in isolation, proliferation is greater when the cells are flattened than when they remain rounded.

PG synthesis in collagenase-treated explants was greater than in control tissue. This effect was only detected when using the higher concentration of collagenase. This may be a response to the loss of matrix from around chondrocytes. When cultured in isolation, chondrocytes re-synthesise a surrounding matrix, but their synthetic rates tend to drop off once matrix has formed around the cells.

In conclusion, the system which we have used to investigate the effect of matrix depletion on articular cartilage has certain drawbacks, in that steady-state metabolism could not be maintained. It would be worthwhile to develop the bovine explant system further in
order to overcome this problem. Whilst recognising the limitations of the system, it would appear that PG depletion has little effect on chondrocyte metabolism in cultured explants of articular cartilage. Disruption of cartilage architecture by collagenase treatment, in contrast, can induce alterations in both DNA and PG synthesis. Whether this effect is a direct response to the loss of collagen from around the cell or due to cell shape changes which can occur when collagen is disrupted is unclear. The finding that PG depletion alone does not alter chondrocyte metabolism is somewhat surprising, as it has been suggested that PG depletion is associated with metabolic changes in arthritic tissue. It must be recognised that in vivo, PG depletion will cause load-related damage to collagen which is unable to withstand compression in isolation. In the system used in this study, however, no load was applied to the tissue and, therefore, cartilage architecture was maintained even when the tissue was depleted of PG. It would be of interest to investigate the effect of PG depletion on loaded cartilage explants, in an attempt to mimic more closely the degenerative changes seen in arthritic tissue.
REFERENCES.
REFERENCES


References.


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