Proteoglycans of cartilage

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Proteoglycans are probably the most important non-fibrillar constituents of connective tissue although little is known about the proteoglycans of connective tissue other than cartilage. Hence this paper is mainly concerned with cartilage proteoglycans.

Proteoglycans are found throughout connective tissue, but those of cartilage have certain features that distinguish them from proteoglycans of other connective tissues. Cartilage is an avascular tissue in which the cells are sparsely distributed in a stiff matrix. Although the content of water is high—about 70% in the cartilage of human femoral condyles (Maroudas *et al.*, 1969)—it is precisely the presence of water in conjunction with proteoglycans and collagen that makes cartilage resilient and elastic. Cartilage may be regarded as a reinforced fibre network which in load-bearing joints has to withstand very high repetitive loads (Freeman and Kempson, 1973).

As was first pointed out by Fessler (1960), a random macromolecular mesh (represented by proteoglycans in this instance) placed within a fibrous network (such as collagen) so that the macromolecules cannot move will impede the flow of interstitial water within the tissue when an external force is applied. Fluid pressure within cartilage rises immediately a load is applied, but as the water is driven out from the loaded area cartilage deforms only gradually because the proteoglycans entrapped in the collagen network impede the flow of interstitial water. Hence the compressive stiffness of cartilage over short intervals is directly correlated with the proteoglycan content measured as glycosaminoglycan (Kempson *et al.*, 1970) (Fig. 1). Proteoglycans exert a swelling pressure that is constantly restrained by the collagen network in which they are entrapped. Maroudas (1975) has calculated the internal osmotic pressure of cartilage of human femoral heads to be about 3.4-3.6 atmospheres. Sorption isotherms also indicate that swelling pressure is mainly attributable to the proteoglycan component of cartilage (Mathews and Decker, 1977).

The relative proportion of collagen to proteoglycan and other constituents varies in different types of connective tissue and largely determines the physical characteristics of the tissue. Collagen

accounts for about half the dry weight of full-thickness articular cartilage, but the amount decreases with depth from the articular surface (Maroudas *et al.*, 1969; Muir *et al.*, 1970). Conversely the proteoglycan content varies approximately inversely with the collagen content and there is a topographical variation in the overall composition of articular cartilage that appears to be characteristic of the individual (Maroudas *et al.*, 1969; Muir *et al.*, 1970; Kempson *et al.*, 1973). The inverse relationship of proteoglycan and collagen contents is also clearly seen in human intervertebral discs (Adams *et al.*, 1977).

General structure of proteoglycans

The sulphated glycosaminoglycans such as chondroitin sulphate, keratan sulphate, or dermatan sulphate are present throughout connective tissue as proteoglycans in which the glycosaminoglycan chains

Fig. 1 Variation of creep modulus (that is, stiffness of cartilage) with total glycosaminoglycan content (from Kempson *et al.*, 1970).

are attached at one end to a protein core. In cartilage proteoglycans as many as 50-100 chondroitin sulphate chains are attached laterally to a protein backbone which comprises about 10-15% of the weight of the molecule. Since the average chain weight of chondroitin sulphate is 15-20 000, cartilage proteoglycans have molecular weights of about 1.3×10^6 daltons (Eyring and Yang, 1968; Luscombe and Phelps, 1967; Pasternack *et al.*, 1974). The general structure, shown diagrammatically in Fig. 2, was proposed by Mathews and Lozaityte (1958) and by Partridge *et al.* (1961). It is consistent with the effects of proteolytic enzymes, to which proteoglycans are particularly vulnerable (Muir, 1958), because when even a few peptide bonds are cleaved the whole molecule falls apart. The electron microscopical appearance of single molecules, spread and visualised by a special technique (Rosenberg *et al.*, 1970a) also agrees with this general structure.

Fig. 2 Diagram of proteoglycan molecule. HA = hyaluronic acid. CS = chondroitin sulphate. KS = keratan sulphate.

In addition to chondroitin sulphate cartilage proteoglycans contain fewer but variable numbers of keratan sulphate chains attached to the same core protein (Seno *et al.*, 1965; Tsiganos and Muir, 1967; Heinegård and Gardell, 1967; Hoffman *et al.*, 1967; Hascall and Riolo, 1972; Heinegård, 1972). In any given cartilage proteoglycans are heterogeneous and exhibit a range of chemical composition and molecular size. Chondroitin sulphate and all sulphated glycosaminoglycans with the exception of keratan

sulphate are attached to protein via a trisaccharide sequence of neutral sugars (Fig. 3) in which xylose is glycosidically attached to the hydroxyl group of serine residues on the core protein. The complete structure of the linkage region was established by the work of Rodén (Rodén and Armand, 1966; Rodén and Smith, 1966; Helting and Rodén, 1968). Xylose, which had not previously been found in animal polysaccharides, is important in co-ordinating the synthesis of glycosaminoglycan chains with the synthesis of core protein.

CHONDROITIN SULPHATE

Chondroitin sulphate consists of repeating disaccharide units of glucuronic acid and N-acetylgalactosamine (Fig. 4). There are about 25-30 such units in the average chain of chondroitin sulphate with almost one sulphate group per disaccharide in one of two isomeric positions. The distribution of sulphate residues along the chains is not uniform, however, as there are fewer in the vicinity of the linkage of carbohydrate to protein (Wasteson and Lindahl, 1971). In chondroitin 4-sulphate the sulphate is attached to C₄ and in chondroitin 6-sulphate to C₆ of the galactosamine residues (Fig. 4). Both forms occur in articular cartilage and chondroitin 6-sulphate gradually increases with age (Hjertquist and Wasteson, 1972) in adult human articular cartilage (Mankin and Lippiello, 1971; Lust and Pronsky, 1972; Hjertquist and Lemperg, 1972; Lemperg *et al.*, 1974). The proportions of each isomer may be deduced from the effects of specific degradative enzymes, known as chondroitinases. The results suggest that the chondroitin sulphate isomers are not present as separate chains but that the sulphate groups occupy one or other isomeric position along the same chain (Mourão and Dietrich, 1973; Seno *et al.*, 1975; Murata and Bjelle, 1977).

The biological significance of the position of the sulphate group is not known. Both chondroitin sulphate isomers display highly ordered helical conformations when stretched films of these compounds are examined by x-ray fibre analysis. In chondroitin 6-sulphate, however, the sulphate groups project further from the chain than in chondroitin 4-sulphate (Isaac and Atkins, 1973; Atkins, 1977). Chondroitin

Fig. 3 Trisaccharide sequence of neutral sugars that link glycosaminoglycan chains such as chondroitin sulphate to serine residues of the core protein.

Fig. 4 Repeating disaccharide units of (a) chondroitin 4-sulphate, and (b) chondroitin 6-sulphate.

6-sulphate may therefore interact more strongly than chondroitin 4-sulphate with the basic groups of collagen and other proteins.

KERATAN SULPHATE

Keratan sulphate is found only in proteoglycans of cartilage, intervertebral discs, and cornea. It contains no uronic acid. It consists essentially of disaccharide repeating units of N-acetylglucosamine and galactose (Fig. 5), and hence molar ratios of glucosamine:galactosamine may be used to determine the relative proportions of keratan sulphate and chondroitin sulphate in isolated proteoglycans and, less precisely, in whole cartilage. Keratan sulphate is much more variable than chondroitin sulphate both in chain length and in the degree of sulphation (reviewed by Muir and Hardingham, 1975). Skeletal keratan sulphate, also called keratan sulphate II, is distinct from corneal keratan sulphate (keratan sulphate I) in the way that it is linked to protein (Seno *et al.*, 1965), in degree of sulphation, and in being more variable in structure (Mathews and Cifonelli, 1965). It is linked to protein through terminal galactosamine residues attached to hydroxyl groups of threonine and serine (Bray *et al.*, 1967; Tsiganos and Muir, 1967; Hopwood and Robinson, 1974).

The sulphate groups are located on C₆ of half the galactose and more than half the glucosamine residues (Bhavanandan and Meyer, 1968). The chains of skeletal keratan sulphate are shorter and more variable in length than are chondroitin sulphate chains, having weight-average molecular weights of 5-10 000 corresponding to about 13 disaccharide repeating units (Hascall and Riolo, 1972; Robinson and Hopwood, 1973). There is an excess of galactose

Fig. 5 Repeating disaccharide unit of keratan sulphate.

over glucosamine (Gregory and Rodén, 1961; Bhavanandan and Meyer, 1967) and there are branch points along the chain where extra galactose is attached. Small amounts of fucose (Bhavanandan and Meyer, 1968) and sialic acid (Toda and Seno, 1970; Hascall and Riolo, 1972) are also present in terminal positions.

Extraction and heterogeneity of proteoglycans

Extraction procedures developed for one kind of cartilage are not necessarily applicable to all kinds and proteoglycans are much more difficult to extract from other types of connective tissue. Much of the earlier work on proteoglycans was initiated by Schubert and his associates (Gerber *et al.*, 1960; Pal and Schubert, 1965; Pal *et al.*, 1966), who employed high-speed homogenisation to achieve efficient extraction when about 65% of the proteoglycan was extracted from bovine nasal cartilage.

High shearing forces degrade polymers of large molecular weight (Harrington and Zimm, 1965) and hence methods employing high-speed homogenisation have been superseded and replaced by dissociative extraction procedures first introduced by Sajdera and Hascall (1969), who examined systematically the effect of ionic strength and type of salt on the efficiency of extraction. The most effective solutions were shown to be 2M CaCl₂, 3M MgCl₂, and 4M guanidinium chloride. The extracting efficiency of different salts is related to their degree of solvation (Mason and Mayes, 1973). About 80-85% of the proteoglycans are extracted from shredded bovine nasal cartilage without homogenisation of the tissue by 4M guanidinium chloride. But this is less effective with articular cartilage (Rosenberg *et al.*, 1973)—particularly that of adult and elderly individuals—which needs to be ground up and briefly pulverised in liquid nitrogen for 15 seconds, when 80-85% of the proteoglycans may be extracted (Bayliss and Ali, 1978a).

Attempts to prepare discrete fractions of proteoglycans by a variety of methods have been unsuccessful. It seems that in a given cartilage there is a population of proteoglycans exhibiting a range of molecular size and chemical composition that is

characteristic of the tissue, articular cartilage being no exception (Hardingham and Muir, 1974a; Lohmander, 1975; Hardingham *et al.*, 1976; Roughley and Mason, 1976; Rosenberg *et al.*, 1976; Heinegård, 1977).

When articular cartilage is extracted sequentially without high-speed homogenisation proteoglycans extracted at later steps tend to be richer in keratan sulphate and protein, although discrete fractions are not obtained (Brandt and Muir, 1971b; Šimůnek and Muir, 1972; Brandt, 1974). Operationally, three classes of proteoglycan may be distinguished, and although each class is itself heterogeneous the range of variation within a class is narrower.

A minor fraction of proteoglycans may be extracted with salt solutions of physiological ionic strength, whereas the majority need to be extracted with 'dissociating' solvents such as 4M guanidinium chloride. The proteoglycans that resist extraction vary in amount roughly with the collagen content of cartilage (Šimůnek and Muir, 1972). Studies of Smith *et al.* (1967), using high resolution electron microscopy and bismuth nitrate to stain proteoglycans in whole articular cartilage, showed that while most of the proteoglycans did not appear to be bound to collagen some were attached transversely to the fibres at two distinct positions along the 64 nm period. This may represent the inextractable fraction of proteoglycans.

Proteoglycans that are extracted at low ionic strength have relatively low molecular weights of about 2.3×10^5 (Tsiganos and Muir, 1969) and contain less protein and keratan sulphate than the majority (Brandt and Muir, 1969, 1971a; Šimůnek and Muir, 1972; Mayes *et al.*, 1973; Hardingham and Muir, 1974a). Using proteinase inhibitors during the extraction and purification, Pearson and Mason (1977) conclude that proteoglycans of low molecular weight do not arise from larger proteoglycans by breakdown due to the action of proteinases during the extraction. Biosynthesis experiments both *in vitro* (Hardingham and Muir, 1972b) and *in vivo* (Lohmander, 1977) also suggest that these proteoglycans are not degradation products nor precursors of larger proteoglycans. Such low molecular weight proteoglycans may be produced by mesenchymal cells in general and may not be characteristic of differentiated chondrocytes, since they are formed by prechondrogenic cells (De Luca *et al.*, 1977). Moreover, bromodeoxyuridine, which prevents differentiation of mesenchymal cells and matrix formation (Abbott and Holtzer, 1968) arrests the synthesis of proteoglycans typical of cartilage but not the synthesis of the low molecular weight proteoglycans (Dorfman *et al.*, 1975).

Proteoglycans that are phenotypic of cartilage are

of much higher molecular weight—about $1.2.5 \times 10^6$ daltons—and contain more protein and more keratan sulphate than proteoglycans extracted at low ionic strength or those in other kinds of connective tissue.

Purification of proteoglycans by equilibrium density gradient centrifugation and dissociation of aggregates

Equilibrium density gradient centrifugation in caesium chloride was first used to purify proteoglycans by Franek and Dunstone (1966). In this method molecules are separated according to their buoyant density in a concentrated caesium chloride gradient. Since the buoyant density of carbohydrates, particularly of polyanionic glycosaminoglycans, is much higher than that of proteins, molecules may be separated according to differences in the proportions of carbohydrate to protein. Those containing the most carbohydrate separate at the bottom of the gradient while those richer in protein separate towards the top at lower densities.

The usefulness of this method was examined by Hascall and Sajdera (1969) for the purification of nasal cartilage proteoglycans. These contain about 90% of glycosaminoglycans and have buoyant densities of about 1.8 g/ml, whereas contaminating proteins such as collagen have buoyant densities around 1.3 g/ml and may be removed at the top of the gradient (Fig. 6). Proteoglycans of articular cartilage are richer in protein than those of nasal septum and lower starting densities must be employed to enable the majority to be separated at the bottom of the gradient (Rosenberg *et al.*, 1973; Rosenberg, 1974; McDevitt and Muir, 1975). Density gradient centrifugation in caesium chloride is now generally carried out in two stages, the first under 'associative' conditions in 0.5M guanidinium chloride to purify the proteoglycans, the second under 'dissociative' conditions in 4M guanidinium chloride to dissociate and separate the constituents of proteoglycan aggregates.

A unique feature of cartilage proteoglycans is their ability to form multimolecular aggregates of very high molecular weight, of the order of 50 million. Detailed evidence of aggregation of highly purified proteoglycans was produced by Hascall and Sajdera (1969), although it had been proposed much earlier (Mathews and Lozaityte, 1958). It was only with the development of efficient non-disruptive methods of extraction and purification by density gradient centrifugation that unequivocal results were obtained. Hascall and Sajdera (1969) showed that proteoglycans extracted from bovine nasal cartilage with dissociating solvents such as 4M guanidinium chloride, dialysed to low ionic strength, and purified

Fig. 6 Equilibrium density gradient centrifugation in caesium chloride. Associative conditions (A). Starting density usually 1.6 g/ml or less. Dissociative conditions (D). Lower fraction from associative gradient mixed with equal volume of 7.5 M guanidinium chloride and starting density adjusted to 1.5 g/ml with caesium chloride. Separation of molecules of decreasing size and buoyant density depicted below.

by caesium chloride density gradient centrifugation consisted of fast and more slowly sedimenting components in the analytical ultracentrifuge. When subjected to a second density gradient centrifugation under *dissociative* conditions in the presence of 4M guanidine hydrochloride about 25% of the protein, originally associated with the proteoglycan, now separated at the top of the gradient; the fast sedimenting component was no longer seen in the analytical ultracentrifuge. This showed that aggregation was not a simple self-association but involved binding to other specific non-proteoglycan components.

On dissociative density gradient centrifugation most proteoglycans separate at the bottom of the gradient but a few of progressively diminishing size are distributed throughout the gradient, the smallest being at the top (Fig. 6). In general, chondroitin sulphate content increases with molecular size whereas protein and keratan sulphate contents vary inversely with molecular size so that the smallest

proteoglycans contain about 30% of protein, which is about three times more than in the largest proteoglycans. The amino-acid composition of the proteoglycans changes through the gradient. Cysteine, methionine, and aspartic acid contents increase with protein content and diminishing size, whereas serine and glycine contents decrease (Rosenberg *et al.*, 1976). As chondroitin sulphate chains are attached to serine residues on the core protein and since the sequence Se-Gly is necessary for recognition by the xylosyltransferase that initiates the synthesis of chondroitin sulphate chains (Baker *et al.*, 1972), there will necessarily be more serine and glycine in the larger molecules that contain more chondroitin sulphate.

The pH of extraction affects the proportion of aggregates finally obtained, which is maximal at pH 4.5 (Hardingham and Muir, 1974a). The proportion of aggregates is much reduced by the action of proteinases during the extraction and purification procedure so that inhibitors of cathepsins and neutral

proteinases are now generally added during extraction and dialysis (Oegema *et al.*, 1975). Such inhibitors should be added particularly in situations where proteinases are likely to be active.

Role of hyaluronic acid in proteoglycan aggregation

It was at first thought that proteoglycans were linked together into aggregates by interaction with a protein, referred to as 'protein-link', that separated at the top of the dissociative gradient (Hascall and Sajdera, 1969; Rosenberg *et al.*, 1970b; Rosenberg *et al.*, 1973). It has since been recognised, however, that aggregation depends upon a highly specific interaction of proteoglycans with hyaluronic acid, discovered by Hardingham and Muir (1972a).

The interaction with hyaluronate appears to be unique to cartilage proteoglycans and is entirely specific to hyaluronate. No other polyanions, even close isomers of hyaluronate such as chondroitin (that is, desulphated chondroitin sulphate), interact (Hascall and Heinegård, 1974b) and divalent cations are not required (Hardingham and Muir, 1972a). The interaction with hyaluronate leads to a large increase in viscosity and hydrodynamic size (Hardingham and Muir, 1972a; Hascall and Heinegård, 1974b) which enables the stoichiometry of the interaction to be examined by viscometry and gel chromatography (Hardingham and Muir, 1972a). Proteoglycans do not cross-link hyaluronate chains and hence possess only a single binding site. In cartilage where there is an excess of proteoglycan over hyaluronate a large number of proteoglycan molecules are bound to a single chain of hyaluronate which can bind as much as 250 times its weight of proteoglycan. Using average molecular weights for proteoglycans and hyaluronate of 2.5×10^6 and 5×10^5 respectively, a model for the complex was deduced (Fig. 7). It was calculated that at maximum packing each proteoglycan molecule would occupy about 20 nm of the length of the hyaluronate chain (Hardingham and Muir, 1974b).

Oligosaccharides of hyaluronate of the size of decasaccharides or larger compete strongly with hyaluronate; smaller oligosaccharides do not (Hardingham and Muir, 1973; Hascall and Heinegård, 1974b). The binding of proteoglycan thus involves only 5 nm of the hyaluronate chain. The packing of proteoglycans along the hyaluronate chain is less close because of the steric hindrance of the numerous chondroitin sulphate side chains of the proteoglycans. When these are mostly removed by chondroitinase digestion the resulting core-proteins may be packed five times more closely along the hyaluronate chain (Hascall and Heinegård, 1974b). This also shows that

the chondroitin sulphate chains themselves play no part in the interaction.

When packed at high density proteoglycan molecules must lie perpendicular to the hyaluronate chain, so that the binding site must be at one end of the core protein. The hyaluronate chain thus acts as a thread holding together numerous proteoglycan molecules (Fig. 7). Electron micrographs of aggregates, published before the involvement of hyaluronate was known (Rosenberg *et al.*, 1970a), are consistent with this interpretation.

The hyaluronate-binding region of the core protein has 5-7 intramolecular disulfide bridges (Hardingham *et al.*, 1976) which maintain its tertiary structure. Reduction of disulfide bonds prevents aggregation (Hascall and Sajdera, 1969), and when proteoglycans are reduced and alkylated interaction with hyaluronate is abolished without change in molecular size or in protein content (Hardingham *et al.*, 1976). The ability to bind to hyaluronate is remarkably resistant to denaturation by heat, and, on re-oxidation of the reduced proteoglycan, loss of interaction is largely reversible (Hardingham *et al.*, 1976). It is, however, very sensitive to specific chemical modification of basic and aromatic amino-acids. Lysine and arginine residues participate directly in the interaction but tryptophan only indirectly (Hardingham *et al.*, 1976). When bound to hyaluronate these lysine residues are partially

Fig. 7 Model of proteoglycan-hyaluronic acid complex. Dimensions were deduced from the stoichiometry of the interaction (Hardingham and Muir, 1974b).

protected from chemical substitution (Heinegård and Hascall, 1977).

The exactness of the conformation of the binding site implies an equally exact conformation of the polar groups on hyaluronic acid. Binding of hyaluronate oligosaccharides to proteoglycan in competition with hyaluronate is abolished when less than 40% of the carboxyl groups on the oligosaccharides are modified or when these are displaced from the pyranose rings by three atoms (Christner *et al.*, 1977). Since chondroitin does not compete with hyaluronate, N-acetylglucosamine residues are also essential and cannot be substituted by N-acetylgalactosamine residues (Hascall and Heinegård, 1974b). Together these results indicate that the effective binding site is of limited size and of precise shape enabling the maximum number of subsite interactions to take place in a small area of the molecule.

The hyaluronate binding region may be obtained by cyanogen bromide cleavage of dissociated proteoglycans (Heinegård, 1977) or by mild tryptic digestion of chondroitinase-treated aggregates (Heinegård and Hascall, 1974a). This region lacks chondroitin sulphate and has a different amino-acid composition from the whole core protein. About 60% of the remainder is accounted for by serine, glycine, proline, and glutamic acid in roughly equimolar proportions (Heinegård and Hascall, 1974a; Heinegård, 1977).

It is assumed that although proteoglycans are extremely heterogeneous in composition all those that are able to interact with hyaluronate possess the same hyaluronate binding region as an invariant part of the molecule. The region of the core protein that bears the glycosaminoglycan chains is thought to be variable in length, and to this different proportions of chondroitin sulphate and keratan sulphate are attached (Heinegård and Hascall, 1974a; Hardingham *et al.*, 1976; Heinegård, 1977). Electron microscopical studies of articular cartilage support this idea and show molecules varying in length from 100–400 nm (Rosenberg *et al.*, 1976; Swann *et al.*, 1976). Proteoglycans that interact with hyaluronate thus form a series in which the hyaluronate binding region is present in molecules of all sizes (Fig. 8) since the ability to interact with hyaluronate is not restricted to any particular size of proteoglycan (Swann *et al.*, 1976).

The hyaluronate binding region represents a greater proportion of the total molecule in smaller proteoglycans and hence the carbohydrate:protein ratio is less and the buoyant density in caesium chloride lower as the size decreases. Proteoglycans of diminishing size therefore separate at decreasing buoyant density in the dissociative gradient. In the

Fig. 8 Three sizes of proteoglycan showing constant hyaluronate binding region and shorter chondroitin sulphate binding region with decreasing size.

smallest proteoglycans the hyaluronate binding region represents much of the total protein (Heinegård, 1977) so that the amino-acid composition (Rosenberg *et al.*, 1976) resembles that of the hyaluronate binding region itself as isolated by partial degradation of large proteoglycans (Heinegård and Hascall, 1974a).

The lengths of chondroitin sulphate chains do not vary significantly among proteoglycans of non-articular cartilage of different buoyant density (Heinegård, 1977), but the lengths decrease appreciably with age in proteoglycans of human knee cartilage (Hjertquist and Wasteson, 1972). The average molecular weight was shown to decrease from 20 000 in young individuals to 16 000 in adult and aged persons.

Partial degradation experiments with proteolytic enzymes suggest that the chondroitin sulphate chains are located along the core protein in groups or clusters of up to 8 chains (Heinegård and Hascall, 1974b), and in proteoglycans of lower buoyant density there appear to be fewer clusters and more single chains (Heinegård, 1977). From the results of partial degradation experiments Heinegård and Hascall (1977) have proposed that most of the keratan sulphate is attached to the core protein near the hyaluronate binding region of the molecule. The preponderance of keratan sulphate in this position would explain why smaller proteoglycans that interact with hyaluronate contain more protein and keratan sulphate than larger proteoglycans.

The keratan sulphate content of articular cartilage and that of all extracted proteoglycan fractions increases with age (Šimůnek and Muir, 1972; Bjelle,

1975; Sweet *et al.*, 1977; Bayliss and Ali, 1978a; Inerot *et al.*, 1978) but the function of keratan sulphate is not known. It is not essential for binding to hyaluronate since proteoglycans from rat chondrosarcoma lack keratan sulphate but are nevertheless capable of aggregation (Oegema *et al.*, 1975).

Role of link protein in aggregation

The interaction of proteoglycans with hyaluronate is an equilibrium which lies well in favour of complex formation under physiological conditions of ionic strength, temperature, and pH (Hardingham and Muir, 1972a, 1975). But the complex is unstable in the ultracentrifuge, unlike the aggregate (Gregory, 1973) which contains a third component, the 'protein-link', that accounts for about a quarter of the protein of the aggregate (Hascall and Sajdera, 1969). The protein link appears to function in stabilising the proteoglycan-hyaluronate complex so that it is no longer in equilibrium with its dissociation products. This is illustrated by the fact that aggregates are unaffected by oligosaccharides of hyaluronate which dissociate the proteoglycan-hyaluronate complex (Hascall and Heinegård, 1974b) (Fig. 9). The protein-link binds to proteoglycan (Caterson and Baker, 1978) but it does not promote aggregation on its own (Tsiganos *et al.*, 1972). It also binds to hyaluronate in the absence of proteoglycan (Oegema *et al.*, 1977). It appears to be present in aggregates in 1:1 ratio with proteoglycan, as deduced from partial degradation of aggregates (Heinegård and Hascall, 1974a). Two closely related link proteins have been isolated with molecular weights of 47 000 and 51 000 which give the same peptides on CNBr cleavage (Baker and Caterson, 1977).

Proteoglycan aggregation as currently envisaged, with the participation of three components, is depicted in Fig. 10. The size of a given aggregate will thus depend on the size of proteoglycan molecules but principally on the length of the hyaluronate chain and on the number of proteoglycans attached to it.

The biological function of proteoglycan aggregation is unknown, but as it is apparently restricted to the proteoglycans of cartilage its role is presumably peculiar to the function of cartilage. The size of aggregates immobilises them very effectively in the collagen network and aggregated proteoglycans must first be dissociated before they can be extracted. Aggregates may be less compressible than monomers and may therefore make a greater contribution to the compressive stiffness of cartilage.

Aggregates are more resistant to attack by proteinases, since controlled partial degradation is possible only with proteoglycan aggregates whereas

Fig. 9 Effect of hyaluronate oligosaccharides on viscosity of proteoglycan aggregates and of proteoglycan-hyaluronate complex in 0.5 M guanidinium chloride at 30°C (Hardingham, unpublished results).

monomers are completely broken down (Hascall and Heinegård, 1974a; Heinegård and Hascall, 1974a). Aggregates may therefore be catabolised more slowly. They may also indirectly play some part in regulating proteoglycan synthesis by chondrocytes, since free hyaluronic acid, but not that bound up in aggregates, inhibits proteoglycan synthesis (Wiebkin *et al.*, 1975).

Metabolic control mechanism

The synthetic activity of chondrocytes is influenced by their immediate surroundings and they respond to losses of proteoglycans from their matrix by increased synthesis. When returned to normal medium after exposure to papain (Bosmann, 1968) or hyaluronidase (Fitton-Jackson, 1970) embryonic chick cartilage in organ culture responded by increased synthesis, so that the loss was replenished within a few days (Hardingham *et al.*, 1972). Conversely, Handley and Lowther (1977) found that 5-10 mg/ml of proteoglycans added to the medium of chick embryonic chondrocyte cultures considerably depressed proteoglycan synthesis. Much lower levels of hyaluronate significantly reduced proteoglycan synthesis by chondrocytes from cartilage of adult pig (Wiebkin and Muir, 1973) or embryonic chick (Toole, 1973; Solursh *et al.*, 1974; Handley and Lowther, 1976). The effect was specific to hyaluronate and involved material on the cell membrane that was destroyed by trypsin but not by chondroitinase

Fig. 10 Diagram of aggregation.

(Wiebkin and Muir, 1975). It has been suggested that either the synthesis of core protein or the first step in chondroitin sulphate chain synthesis may be inhibited (Handley and Lowther, 1976).

Both collagen and proteoglycans have been shown by autoradiography to be synthesised by chondrocytes, but how the syntheses are co-ordinated is not known. Over short periods of time *in vitro* the synthesis of one was unaffected by inhibition of the synthesis of the other (Dondi and Muir, 1976) and hence the two processes are not closely inter-dependent.

Degradative processes

During normal turnover the catabolism of proteoglycans and other constituents of cartilage must be brought about by cells within the tissue. The structure of proteoglycans makes them particularly vulnerable to attack by proteolytic enzymes (Fig. 2) and different proteolytic enzymes reduce the viscosity of proteoglycan solutions to different degrees (Muir, 1958). Many lysosomal proteinases are now well characterised and have been shown to degrade proteoglycans to fragments of different size (Morrison *et al.*, 1973; Malmud and Janoff, 1975; Keiser *et al.*, 1976). However, proteinases active at neutral pH are likely to be principally involved in normal turnover of proteoglycans (Sapolsky *et al.*, 1974; Sapolsky *et al.*, 1976).

Cathepsin B, a neutral thiol proteinase, is present in human articular cartilage in high levels in juveniles but fall to low levels in adults (Bayliss and Ali, 1978b). In rheumatoid arthritis inflammatory cells infiltrate the joint; they include polymorphonuclear leucocytes which contain neutral proteinases (reviewed by Harris and Cartwright, 1977) that degrade proteoglycans in whole cartilage (Malmud and Janoff, 1975). Cartilage is normally impermeable to large solutes such as proteins (Maroudas, 1973) because proteoglycans exclude large molecules from

their domains. But when degraded by proteolytic enzymes their size is reduced so much that they can diffuse out from the cartilage, so allowing penetration of proteolytic enzymes and further degradation of the tissue. The collagen fibres are then exposed to the effects of collagenase released from inflammatory cells.

How far cathepsin D participates in normal turnover is not clear. During autolysis (Dingle *et al.*, 1972) or exposure to excess vitamin A (Poole *et al.*, 1974), which stimulates the release of lysosomal enzymes, the loss of proteoglycans from cartilage matrix is attributable to the action of cathepsin D, whose extracellular localisation has been shown by immunofluorescence using specific antisera to cathepsin D (Poole *et al.*, 1974).

Normal cartilage contains proteinase inhibitors, among which are low molecular weight cationic proteins (Kuettner *et al.*, 1974; Kuettner *et al.*, 1976; Kuettner *et al.*, 1977) and other specific inhibitors (Roughley *et al.*, 1978). Probably such proteinase inhibitors would normally modulate the activity of proteinases. These may be released not only from inflammatory cells but also from cells of the soft tissues of the joint, particularly in the presence of immune complexes (Fell and Barratt, 1973; Poole *et al.*, 1973). In such situations the inhibitors may be overwhelmed by the influx of proteinases. Moreover, in organ cultures of normal cartilage chondrocytes are stimulated to degrade their surrounding matrix by some factor that emanates from synovial tissue (Fell and Jubb, 1977).

Partially degraded proteoglycans which diffuse out from cartilage and enter the circulation are rapidly removed by the liver and completely broken down (Wood *et al.*, 1973). The liver is able to catabolise all the proteoglycan released during normal turnover and only a very small proportion of the total reaches the urine (Wood *et al.*, 1973).

Osteoarthritis

In osteoarthritis in man and domestic animals the proportion of aggregated proteoglycans is less than normal (Brandt *et al.*, 1976; Palmoski and Brandt, 1976; Inerot *et al.*, 1978) and the monomeric proteoglycans are smaller and more heterogeneous (Sweet *et al.*, 1977; Inerot *et al.*, 1978) while the chondroitin sulphate chains are abnormally short and heterogeneous (Hjertquist and Wasteson, 1972). Both in whole cartilage and in the proteoglycans extracted from osteoarthrotic cartilage the proportion of chondroitin sulphate relative to keratan sulphate is invariably higher than normal (Mankin and Lippiello, 1971; Brandt, 1974; McDevitt and Muir, 1974, 1975, 1976; Palmoski and Brandt, 1976;

Brandt *et al.*, 1976; Sweet *et al.*, 1977; Bayliss and Ali, 1978a) but the significance of this change is unknown. The rates of incorporation of ^3H -thymidine and $^{35}\text{SO}_4$ are closely correlated (Mankin and Lippiello, 1971) and Mankin *et al.* (1971) have suggested that in osteoarthritis chondrocytes revert to a chondroblastic phase, undergo cell division, and start to synthesise proteoglycans characteristic of immature cartilage in that these contain less keratan sulphate relative to chondroitin sulphate than proteoglycans of mature cartilage of comparable age.

Degradative enzymes produced by chondrocytes themselves may play some part in the destruction of cartilage. The activity of cathepsin B was found to be considerably raised in specimens of osteoarthrotic cartilage of human femoral heads removed for total hip replacement compared with normal cartilage of comparable age taken from fractured femoral heads (Bayliss and Ali, 1978b). Osteoarthrotic changes occur first in certain discrete areas of the femoral head (Byers *et al.*, 1970), and in these areas the activity of cathepsin D was much higher than in visibly normal surrounding areas (Ali and Evans, 1973).

The changes seen in osteoarthritis and in mineralisation of cartilage are similar in certain respects. In both cases proteoglycan content decreases (Lohmander and Hjerpe, 1975), the qualitative changes in proteoglycans are similar, and alkaline phosphatase activity is greatly raised (Ali and Evans, 1973; Howell *et al.*, 1976), particularly in osteophytic cartilage (Bayliss and Ali, 1978b). Alkaline phosphatase activity in human articular cartilage is largely in matrix vesicles (Ali, 1976), and vesicles containing hydroxyapatite crystallites are present in the deep zone of osteoarthrotic human cartilage. Calcification of this zone would then allow the advance of a mineral front and remodelling of subchondral bone (Ali, 1977). It is notable that loss of aggregation is a feature of osteoarthritis and that the growth of apatite crystals is thought to be inhibited by proteoglycan aggregates (Cuervo *et al.*, 1973). It is not yet certain, however, how far qualitative changes in proteoglycans and changes in mineral metabolism are strictly interdependent.

The initial stages of osteoarthritis cannot be studied in the natural disease because it is impossible to know when it began. Using an experimentally induced osteoarthritis in the dog that closely resembles the natural disease it has been possible to study the earliest biochemical changes that precede the appearance of lesions, since these develop in the same area of the tibial condyle in each animal. This region can therefore be sampled before lesions appear (McDevitt and Muir, 1974, 1975, 1976; McDevitt *et al.*, 1977). Increased hydration of the cartilage was

a primary change and consequently the proteoglycans were more extractable. Profound metabolic changes took place and proteoglycans of different chemical composition were produced. It appeared that there were three phases in the early development of the disease (Muir, 1977). Only by the third phase, when osteophytes first appear at the joint margins, would the disease be recognised as osteoarthritis by its gross and microscopic appearances, although at this stage the disease is far less advanced than that seen in human clinical osteoarthritis.

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