Occasional Survey

Biochemistry of articular cartilage

Nature of proteoglycans and collagen of articular cartilage and their role in ageing and in osteoarthritis

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The cartilage which covers the articular surfaces of bones of vertebrate animals has two main functions: the limitation of stress applied to the bone extremities and the provision of the smooth surfaces necessary for the joint to function as an efficient bearing (Kempson, Spivey, Freeman, and Swanson, 1969). To meet these functional demands the cartilage exhibits a combination of physical properties which are unique to the tissue. Boundary effects, resulting from the interaction of the cartilage surface with synovial fluid, provide the joint with a bearing surface having a very low coefficient of friction. Articulation of a joint produces compressive stresses which arise from both muscle pull and gravity. In the absence of articular cartilage these stresses would be transmitted directly to the bones and would possibly fracture them. The viscoelastic nature of articular cartilage enables it to absorb the energy of these mechanical stresses. When an external stress is applied to the cartilage, the tissue undergoes a slowly progressive deformation. When the external stress is removed the cartilage can restore its original shape. The principal components of articular cartilage are the insoluble fibrous protein collagen and the soluble proteoglycans. A complex organization of collagen, proteoglycans, the fluid environment, and as yet uncharacterized components, endows the tissue with the capacity for reversible deformability, a property essential for its physiological function.

The biochemist is primarily interested in the chemical composition of the tissue, the elucidation of the structure and organization of the constituent macromolecules, the nature of the synthetic and degradative systems in the tissue, and the influence of age and pathological state on these phenomena.

Articular cartilage, as a tissue, has presented considerable difficulties to biochemical investigation. The tissue displays a complex morphology, as yet incompletely understood, and is very resistant to solubili-

zation. For these reasons there is a paucity of biochemical information on articular cartilage compared to other cartilagenous tissues. It should be emphasized that joint cartilage is different in many respects from other cartilagenous tissues and extrapolation of data, as has often been done in the past, is not always valid. Furthermore, variation in joint cartilage composition has been noted between different species and even between different joints of any one subject. It would seem that a high degree of specialization has evolved in articular cartilage.

Composition of the tissue

Mature articular cartilage appears to be totally avascular and aneural and without lymphatic vessels or a limiting membrane (Barnett, Davies, and MacConaill, 1961; Cameron and Robinson, 1958). A characteristic feature of the tissue is the paucity of cells which account for only a small volume of the hydrated cartilage. The degree of hydration and the relative proportions of the various extracellular constituents varies with age and species. The extra-cellular phase is highly hydrated, with the dry weight amounting to only 20–30 per cent. of the wet weight of the tissue. Collagen usually comprises about 60 per cent. and the glycosaminoglycans about 10 per cent. of the dry weight of the adult tissue. The ash content has been estimated to be 6.2 per cent, sulphur 1.3 per cent., sialic acid 0.52 per cent., and total nitrogen about 14 per cent. of the dry weight of the cartilage (Matthews, 1953; Kuhn and Leppelmann, 1957; Bollet and Nance, 1966; Anderson, Ludwig, Harper, and Engleman, 1964; Campo and Tourtellotte, 1967; Maroudas, Muir, and Wingham, 1969). The residual dry weight is comprised of trace amounts of lipid (Collins, Ghadially, and Meachim, 1965; Ghadially, Meachim, and Collins, 1965; Stockwell, 1965), phospholipid (Guarda and Turra, 1967), lysozyme (Kuettner, Guenther, Ray, and

FIG. 1 Diagram of proteoglycan molecule. Site of attachment of keratan sulphate chains and conformation of protein core are at present unknown

Schumacher, 1968), and as yet uncharacterized components which may be glycoprotein in composition.

PROTEOGLYCANS

Proteoglycan (or proteinpolysaccharide) is the term applied to a family of compounds, the basic structure of which may be represented by a single protein 'core' to which chains of glycosaminoglycans are covalently attached (Fig. 1). The major glycosaminoglycan in articular cartilage is chondroitin-6-sulphate (Mankin and Lippiello, 1971; Lust and Pronsky, 1972). Smaller amounts of keratan sulphate and chondroitin-4-sulphate also exist. These compounds have been renamed (Jeanloz, 1960) and a list of the old and corresponding new terms appears in Table I. The elucidation of the molecular structure of the glycosaminoglycans, mainly the work of Meyer and his colleagues, has been extensively reviewed (Muir, 1964; Brimacombe and Webber, 1964; Schiller, 1966; Rosenberg and Schubert, 1970). A summary of the analytical procedures employed in the laboratory manipulation of these compounds has also appeared (Barrett, 1968). The biosynthesis of these compounds, a feature which will not be described in this survey, has been reviewed in detail by Rodén (1970a).

The glycosaminoglycans are long-chain heteropolysaccharides composed of repeating disaccharide units. The disaccharide unit consists of a hexosamine and a non-nitrogenous sugar linked by a glycosidic bond. The structures of chondroitin-4 and 6-sulphate and keratan sulphate are shown in Fig. 2. Chondroitin-4 and 6-sulphate have identical repeating units,

FIG. 2 Structures of disaccharide repeating unit of (a) chondroitin 6-sulphate, (b) chondroitin 4-sulphate, (c) keratan sulphate, (d) hyaluronic acid

except that a sulphate ester group appears on carbon 4 of the galactosamine moiety in the former and carbon 6 in the latter. The structural representation of keratan sulphate is probably an oversimplification, as keratan sulphate preparations contain small amounts of fucose, sialate, galactosamine, mannose, and extra ester sulphate (Mathews and Cifonelli, 1965; Bray, Lieberman, and Meyer, 1967; Bhavanandan and Meyer, 1968).

Hexosamine estimations are indicative of the total glycosaminoglycan (chondroitin sulphate and keratan sulphate) content of a mixture. Chondroitin sulphate levels are normally determined by measuring the

Table I Nomenclature of glycosaminoglycans (formerly called acidic mucopolysaccharides)

Current term	Old term	Constituent sugars	Acidic groups
Chondroitin 6-sulphate	Chondroitin sulphate C	N-acetylgalactosamine and hexuronic acid	Carboxyl and sulphate
Chondroitin 4-sulphate	Chondroitin sulphate A	N-acetylgalactosamine and hexuronic acid	Carboxyl and sulphate
Keratan sulphate	Kerato sulphate	N-acetylglucosamine and galactose	Sulphate
Hyaluronic acid	—	N-acetylglucosamine and hexuronic acid	Carboxyl

hexuronic acid content. Determination of the relative proportions of galactosamine and glucosamine gives the relative proportions of chondroitin sulphate and keratan sulphate. Neutral sugars, such as xylose and galactose, may be estimated by means of gas liquid chromatography (Ewins, 1972).

Two properties of the glycosaminoglycan chains are important in endowing the intact proteoglycans with special features essential for their biological function. The glycosaminoglycans are polyanions; each carboxylate and each ester sulphate group carries a single anionic charge. Thus, each repeating disaccharide in chondroitin sulphate is associated with two unit negative charges, and in keratan sulphate with one negative charge. These anionic groups are always associated with a counterion, which in the native state is predominantly sodium. Articular cartilage chondroitin sulphate contains approximately 26 repeating disaccharide units which corresponds to a molecular weight of approximately 15,000 (Brandt and Muir, 1969b).

The glycosaminoglycans in cartilage are always bound to protein. Both chondroitin sulphates are linked to the hydroxyl groups (Anderson, Hoffman, and Meyer, 1965) of serine residues (Muir, 1958) in the protein core. The serine is bridged to the regular repeating units of the chondroitin sulphate by a specific oligosaccharide with a sugar sequence atypical of the rest of the chain. Rodén and his co-workers established the sequence of this linkage region as o-serine → xylose → galactose → galactose → glucuronic acid (Rodén and Armand, 1966; Lindahl and Rodén, 1966; Rodén and Smith, 1966; Helting and Rodén, 1968). The nature of the non-reducing (*i.e.* terminal) end of the chain remains to be established, but preliminary reports suggest that the terminal sugar is usually a hexosamine (Rodén, 1970b). The distribution of sulphate residues along this chain is not uniform; the chondroitin sulphate contains relatively less sulphate in the vicinity of the carbohydrate-protein linkages than in the more peripheral portions of the chain (Wasteson and Lindahl, 1971). Keratan sulphate appears to be attached through a terminal galactosamine to serine and threonine residues on the protein backbone (Bray and others, 1967).

The properties of the proteoglycan macromolecule are consistent with a comb-like structure (Fig. 1) (Mathews and Lozaityte, 1958; Partridge, Davis, and Adair, 1961; Cessi and Bernardi, 1965). The amino acid sequence and the conformation of the protein 'core' remain to be established. Although optical rotary dispersion and circular dichroism studies led Eyring and Yang (1968) to conclude that the protein backbone had little helical content and no inter-molecular disulphide linkages, Sajdera and Hascall (1969) have postulated a core protein conformation which is sensitive to high-speed homogenization.

It is probable that the 'comb' model for the proteoglycan molecule is a simplification and that at least some regions of the protein core can assume a tertiary configuration. The chondroitin sulphate and keratan sulphate chains are distributed along this backbone although not all serines are involved in the linkages. Recent studies by Mathews (1971) with proteoglycan isolated from bovine nasal septa cartilage indicate that the chondroitin sulphate chains are not uniformly distributed along the protein chain. The chondroitin sulphate chains tended to occur in pairs, each chain of the pair being separated by less than ten amino acids. These 'doublet' regions were separated by a protein core length of approximately 35 amino acids. The distribution of keratan sulphate residues along the protein core is unknown.

The glycosaminoglycan chains, being anchored to the protein core and possessing a high negative charge density, tend to repel one another in solution. A consequence of this is that the number of probable conformations the proteoglycan molecule may assume is considerably reduced. Both the polysaccharide and protein components tend to extend themselves because of the charge repulsion and the proteoglycan attempts to occupy the largest possible domain in solution. Fully hydrated proteoglycans have large Stokes radii and are retained by scintered glass filters of pore size 1.35–1.70 μ and by Millipore filters of 220 m μ mean pore size (Gerber and Schubert, 1964). They are immobilized in 7 per cent. polyacrylamide gels (Barrett, 1966) and some are sufficiently large to be excluded from Sepharose 2B (Tsiganos, Hardingham, and Muir, 1971) or large-pore polyacrylamide-agarose gels (McDevitt and Muir, 1971). In this fashion the proteoglycan acquires an elastic resistance to compression. The elasticity and the large molecular size of the hydrated proteoglycans are important in the functioning of articular cartilage and this feature of the tissue is discussed at the end of the section 'Macromolecular organization in the tissue'.

COLLAGEN

The collagen of articular cartilage is typical of the collagen family of proteins in containing hydroxylysine and large amounts of glycine and hydroxyproline. It differs from skin collagen in its extractability, fibril diameter, and the nature of the α -chains which comprise its molecular structure.

The ultrastructure of articular cartilage collagen has received considerable attention from transmission and scanning electron microscopy. Clarke (1971), in a recent review, has drawn attention to the lack of agreement on fibril diameters and has suggested that tissue preparation methods may be in part responsible for these discrepancies. In particular, an adherent coating of proteoglycan or other non-collagenous substance which is probably removed during aldehyde fixation (Szirmai, 1963) may influence fibril diameter

estimations and obscure fibril striations at higher magnifications. The collagen in the superficial zone, variously reported as parallel bundles (Robinson and Cameron, 1956) or parallel fibrils (Little, Pimm, and Trueta, 1958), displays an orientation which is circumferential. In the subsuperficial zones, while both radial and oblique fibrils may be observed, the predominant arrangement is that of an unbundled fibrillar network with a random orientation (Zelander, 1959; McCall, 1969; Clarke, 1971). The fibril diameter varies with the age (see section on Ageing) and the depth of the section from the articular surface. Although fibrils of less than 10 μm diameter are apparently present in all zones, fibril diameters of 34 nm., 70 to 100 nm., and 140 nm. in the superficial, middle, and deep zone respectively were observed in a transmission electron microscope study by Muir, Bullough, and Maroudas (1970). A typical collagen fibril periodicity of 640 nm. has been extensively reported (Matukas, Panner, and Orbison, 1967; Smith, Peters, and Serafini-Fracassini, 1967; Ruttner and Spycher, 1968; Weiss, Rosenberg, and Helfet, 1968; Muir and others, 1970).

A characteristic feature of articular cartilage collagen is its almost total resistance to solubilization by agents successfully employed in the extraction of skin collagens. Extraction with 5M guanidine-HCl (Miller, van der Korst, and Sokoloff, 1969), 2M CaCl_2 , 2M MgCl_2 or 6M Urea (Strawich and Nimni, 1971) brought into solution less than 3 per cent. of the collagen of the tissue. Furthermore, pathological conditions, such as lameness in pigs or osteoarthritis in dogs, which considerably enhanced the extractability of proteoglycans from articular cartilage, did not markedly affect the ease of solubilization of the collagen fraction (McDevitt and Muir, unpublished).

The low extractability of articular cartilage collagen appears to be due in part to the insolubility of the complex formed between positively charged regions of the collagen molecule and the negatively charged proteoglycans. Solubilization of the collagen fraction is facilitated if the tissue is first incubated with enzymes, such as papain at 4°C., which are considered selectively to degrade the proteoglycans (Strawich and Nimni, 1971). Molecular entanglements and excluded volume effects contribute to the stability of the proteoglycan-collagen complexes (Fessler, 1960; Laurent and Pietruszkiewicz, 1961; Disalvo and Schubert, 1966; Toole and Lowther, 1968; Miller and Matukas, 1969; Steven, Broady, and Jackson, 1969; Strawich and Nimni, 1971; Brandt and Muir, 1971b). The low extractability of articular cartilage collagen is also due to the presence of inter- and intramolecular cross-links which increase with age. Lathyrogens, which inhibit cross-linking, increase the solubilization of the collagen (Glimcher, Seyer, and Brickley, 1969). Furthermore, collagen from new

born and developing canine cartilage is slightly more soluble than that from mature dogs (Lust, Pronsky, and Sherman, 1972). More direct evidence for the presence of a cross-linking cartilage collagen has been provided by Miller (1971). Studies of borohydride-reduced sternal cartilage collagen, which was cleaved at its methionyl residues by cyanogen bromide, showed the presence of an intermolecular cross-link which probably arose through the formation of a Schiff base or aldol condensation product between two hydroxylsyl residues (Miller, 1971).

The monomeric unit of native collagen is a triple-stranded helix with a molecular weight of about 300,000. The individual peptide strands in the helix are called α chains. The degradation of insoluble newborn skin collagen with cyanogen bromide and the subsequent separation and characterization of the peptide fragments has revealed the presence of three genetically distinct α chains in this tissue. These chains have been termed $\alpha 1$ (1) (read 'alpha one, type one'), $\alpha 1$ (11), and $\alpha 2$. It now seems that human skin collagen is a mixture of molecules with the compositions $[\alpha 1 (1)]_2 \alpha 2$ and $[\alpha 1 (11)]_3$. Cartilage collagen is comprised of a different type of α chain which, on cyanogen bromide cleavage, gives different peptides to those from skin collagens. The presence of this chain, termed $\alpha 1$ (11), was originally detected in chick sternal cartilage (Miller and Matukas, 1969) and has subsequently been demonstrated in bovine articular cartilage (Strawich and Nimni, 1971). The soluble bovine cartilage collagen is apparently all of the type $[\alpha 1 (11)]_3$. The cartilage collagen α chain and the $\alpha 1$ (1) chain from skin collagen are similar in both size and charge, as their sedimentation velocities, mobilities in polyacrylamide gel electrophoresis, and elution patterns on CM-cellulose chromatography were identical (Strawich and Nimni, 1971).

Amino acid analyses have shown that there is more hydroxyproline and hydroxylsine and less lysine and proline in articular cartilage collagen compared to skin collagen (Miller and others, 1969; Steven and others, 1969; Strawich and Nimni, 1971). Approximately half the hydroxylsine residues in the cartilage collagen isolated by Strawich and Nimni (1971) were covalently bound to galactose residues. This feature of articular cartilage collagen may be related to the functional requirements of the tissue. Collagens such as lens capsule (Spiro and Fukushima, 1969) or glomerular basement membrane collagen (Kefalides, 1968), which have a relatively high content of hydroxylsine-linked hexose, tend to exhibit a lower degree of morphological organization or to form smaller fibrils. The carbohydrate apparently influences the aggregation properties of the collagen molecule (Spiro, 1970; Strawich and Nimni, 1971). The atypical segment long spacing (SLS) pattern (Trelstad, Kang, Igarashi, and Gross, 1970), the

small fibril diameter, and the random organization in the sub-superficial zones suggest that hydroxylysine-linked carbohydrate participates in the formation of a specialized quaternary structure in articular cartilage collagen.

LIPIDS

Histological and electronmicroscopic studies have revealed a small amount of lipid and phospholipid in articular cartilage (Collins and others, 1965; Ghadially and others, 1965; Stockwell, 1967b; Guarda and Turra, 1967). The total lipid extracted from human articular cartilage during the first three decades of life amounted to less than 1 per cent. of the wet weight of the tissue (Stockwell, 1967b). Extracellular lipid which was concentrated in the superficial zone was demonstrated in adult humeral cartilage (Ghadially and others, 1965). This extracellular lipid tended to be concentrated around the tangentially-oriented chondrocytes of the upper one or two cell layers and often showed membranous structures in the electron microscope. These considerations suggest that at least part of this lipid fraction is located in membrane-bounded matrix vesicles. The presence of these vesicles has been demonstrated in bovine foetal epiphyseal cartilage (Ali, Sajdera, and Anderson, 1970). They have been implicated in the initial calcification of cartilage and possess enzymes which can increase the local concentration of orthophosphate (Ali and others, 1970). Dormant matrix vesicles have been observed in adult articular cartilage (Ali, personal communication).

The phospholipids, which are uniformly distributed in the tangential zone but occur only in the immediate vicinity of cells in the calcified zone of cartilage, consist mostly of sphingomyelin with smaller amounts of lecithin and cephalin (Guarda and Turra, 1967).

Macromolecular organization in articular cartilage

Chemical and histological analysis of articular cartilage has revealed a topographical variation of the collagen, chondroitin sulphate, and keratan sulphate contents in the tissue (Stockwell and Scott, 1965, 1967; Maroudas and others, 1969). The collagen content is highest in the superficial layer and declines with depth from the articular surface. The chondroitin and keratan sulphate contents, however, are lowest in the outermost layer and are higher in the deeper zones in the tissue. A crude inverse correlation exists between the focal contents of collagen and proteoglycans in the cartilage.

The entrapment of the high molecular weight proteoglycan molecules in the insoluble collagen matrix has presented difficulties in their extraction from articular cartilage. An ideal extraction procedure would result in total solubilization without degrada-

tion of the proteoglycans of the cartilage. None of the methods currently available achieves this ideal. There are three different methods of proteoglycan extraction and the type of product obtained is dependent on the method used (Rosenberg, Pal, Beale, and Schubert, 1970).

High-speed homogenization of bovine nasal cartilage in water brought into solution a product which amounted to 55 per cent. of the dry weight of the cartilage (Malawista and Schubert, 1958). Procedures which involved high-speed homogenization of the cartilage have since been termed the 'disruptive method' (Sajdera and Hascall, 1969). High-speed centrifugation separated this type of cartilage extract into two fractions; a major component ('light' protein-polysaccharide or PP-L) which sedimented at 100,000 G. and a 'heavy' component (PP-H) which sedimented at 10,000 G. (Gerber, Franklin, and Schubert, 1960). The PP-L fraction was separated into a further four components (PPL 3, PPL 4, PPL 5, and PPL 6) by centrifugation in high concentrations of potassium acetate and calcium chloride (Pal, Doganges, and Schubert, 1966).

The extraction of cartilage in salt solutions of high concentration without high-speed homogenization is now the most widely applied method for isolating the proteoglycans from the tissue in high yield. A comprehensive investigation by Sajdera and Hascall (1969) established that the nature and concentration of the electrolyte were the critical parameters and that the pH and temperature were less influential in determining the efficiency of the extraction procedure. They found that 4M guanidinium chloride 2M CaCl_2 , 3M MgCl_2 and 6M LiCl were the optimum concentrations of these electrolytes for mobilization of the proteoglycans from cartilage. Extraction of bovine nasal cartilage with 4M guanidine chloride brought into solution 85 per cent. of the uronate of the tissue. This method of extraction, termed the 'dissociative' method (Sajdera and Hascall, 1969), has certain advantages over the 'disruptive' method. The high shear employed in the disruptive method appeared to result in some proteoglycan degradation, as the macromolecules isolated by this procedure had lower average sedimentation coefficients than those isolated by the dissociative method (Sajdera and Hascall, 1969).

Extraction of cartilage with salt solutions of low ionic strength without high-speed homogenization (Tsiganos and Muir, 1969a) brings into solution a small amount of the proteoglycans of the articular tissue (Brandt and Muir, 1969a, b, 1971a; Šimůnek and Muir, 1972a). Approximately 6 per cent. of the uronate of mature porcine articular cartilage was solubilized in 0.15M sodium acetate (Šimůnek and Muir, 1972a). These easily-mobilized proteoglycans have been shown to be smaller in hydrodynamic size than the residual proteoglycans in

the tissue (Brandt and Muir, 1969a, b, 1971a; Šimůnek and Muir, 1972a), and the procedure is particularly valuable where molecular selectivity is a priority.

Two important features of the proteoglycan population of cartilage have been demonstrated in recent years;

- (1) the proteoglycans consist of a series of subpopulations which differ in size and composition;
- (2) Some of these molecules can aggregate in the presence of a 'link' component.

The proteoglycans of articular cartilage consist of a family of closely related compounds which differ in hydrodynamic size, chemical composition, the ease with which they may be extracted from the tissue (Brandt and Muir, 1969a, b, 1971a; Šimůnek and Muir, 1972a), and their gel electrophoretic mobility (McDevitt and Muir, 1971). This heterogeneity has also been demonstrated in other cartilagenous tissues, the proteoglycans of which could also be separated by electrophoresis (Muir and Jacobs, 1967) and equilibrium-density centrifugation (Tsiganos and others, 1971). Sequential extraction of articular cartilage results in mobilization of proteoglycans from the tissue which increase in hydrodynamic size with each successive extract (Brandt and Muir, 1969a, b, 1971a; Šimůnek and Muir, 1972a). The smallest proteoglycans had less protein and a lower keratan sulphate content, as determined by galactosamine/glucosamine molar ratios, than the larger proteoglycans (Brandt and Muir, 1969a, b).

The absence of high shearing forces in the extraction procedure and the fact that lysosomal protease activity was reduced by isolation of the smaller proteoglycans in neutral solution suggest that the heterogeneity was not an artefact of the extraction procedure. Furthermore, pulse-chase experiments *in vitro* with pig laryngeal cartilage slices failed to show a precursor-produce relationship between proteoglycans of different size (Hardingham and Muir, 1972a). Hydrodynamic size difference cannot be attributed to variation in the size of the chondroitin sulphate chains, as the average chain length of these polysaccharide components was the same in the smaller proteoglycans as in the larger (Brandt and Muir, 1969b). The differences in amino acid composition, N-terminal amino acid residues (Tsiganos and Muir, 1969b), and antigenic determinants suggest that the heterogeneity arises from the presence of more than one 'core' protein. Laryngeal and nasal septum proteoglycans, which were separated from glycoproteins by equilibrium density gradient centrifugation (Hascall and Sajdera, 1969) and cleaved of their chondroitin sulphate chains by protease-free hyaluronidase, were separated by gel chromatography into fractions which showed significant differences in amino acid composition and mobility on polyacrylamide gel electrophoresis (Baxter, 1972).

The other important feature of the proteoglycan population, the capacity for some of the proteoglycans to undergo reversible aggregation in the presence of a specific 'link' component, was originally demonstrated in bovine nasal cartilage (Hascall and Sajdera, 1969) and has subsequently been shown in articular cartilage (Rosenberg, 1971b). A 4M guanidinium chloride extract of bovine nasal cartilage was separated by equilibrium-density gradient centrifugation in the presence of 0.5M guanidinium chloride into three major fractions: a collagen fraction at the top of the gradient, a predominantly protein fraction just below the collagen, and a heavy proteoglycan fraction (termed PPC) at the bottom of the gradient (Hascall and Sajdera, 1969). The bottom proteoglycan fraction (PPC) was further separated in a caesium chloride gradient containing 4M guanidinium chloride into one, a light protein and glucosamine rich fraction (GPL or 'glycoprotein link'), and two, a heavy proteoglycan fraction (PGS or 'proteoglycan subunit') which was recovered from the bottom of the gradient (Sajdera and Hascall, 1969; Gregory, Sajdera, Hascall, and Dziewiatowski, 1970). Sedimentation coefficients and viscosity data suggested that the PPC was disaggregated into proteoglycan subunits (PGS) in the presence of 4M guanidinium. This process was found to be reversible and aggregation of the subunits was effected in the presence of the 'glycoprotein link' fraction.

Sedimentation, viscosity, and electron microscopic studies in which bovine nasal cartilage proteoglycans were visualized by coating with cytochrome C suggest that the proteoglycan subunit (PGS) is itself a proteoglycan dimer formed by end-to-end association of the monomeric form (Rosenberg and others, 1970; Rosenberg, Hellmann, and Kleinschmidt, 1970). Another fraction, termed PPL3, which was isolated by ultracentrifugation of the same cartilage extract as the 'subunit', consisted predominantly of monomeric proteoglycans. It would therefore appear that, in bovine nasal cartilage at any rate, two orders of proteoglycan aggregation exist and that the type of the proteoglycan aggregate isolated depends on the nature of the separation procedure employed.

The nature of the moiety, or moieties, which induce aggregation of the proteoglycan subunits remains to be established. A fraction which promoted interaction between the disaggregated proteoglycans from pig laryngeal cartilage was detected in the middle portion of a caesium chloride equilibrium-density gradient and was more than half carbohydrate in composition (Tsiganos, Hardingham, and Muir, 1972). The component responsible for the increase in the hydrodynamic size of the proteoglycans was subsequently isolated and identified as hyaluronic acid (Hardingham and Muir, 1973). This effect, which was produced with amounts of hyaluronic acid as small as 0.01–1.0 per cent., was apparently a specific one and was

accompanied by a large increase in viscosity (Hardingham and Muir, 1972b). Although the proteoglycan aggregates from bovine nasal cartilage (Hascall and Sajdera, 1969) and the proteoglycan-hyaluronic acid complex from pig laryngeal cartilage were similar in the effect of guanidine-HCl concentration and pH on their respective viscosities (Hardingham and Muir, 1972b), they differed in their sedimentation rates in the ultracentrifuge (Hardingham and Muir, 1973). The precise roles of hyaluronic acid and the 'glycoprotein' link in the aggregation of proteoglycans and the effect of age and the pathological state on these phenomena remain to be established.

The high viscosity and large molecular size of the proteoglycans and proteoglycan aggregates reduce their capacity to diffuse through the collagenous fibrillar network of the cartilage. The elastic nature of the proteoglycans and the capacity of these molecules to entrain large volumes of water, an incompressible fluid, in their molecular domains enable the cartilage to absorb the stresses which arise on articulation of the joint. In the absence of cartilage these stresses would be transmitted directly to the bone. The energy of the stress is stored in the cartilage as potential energy and released on removal of the stress. The cartilage regains its original shape on release of this energy, a process in which the elastic nature of the proteoglycans plays a crucial role.

Several models have been proposed to explain the methods whereby the articular cartilage provides a bearing with a very low coefficient of friction and limits the compressive stresses to the underlying bone (Lancet, 1969; Hamerman, 1970). These models are simplifications and a proper understanding of normal cartilage functioning must await further knowledge of the nature and interactions of the macromolecules of the tissue.

Ageing of articular cartilage

Joint cartilage cells during late foetal and early post-natal life serve two purposes. The cartilage adjacent to the synovial cavity contains a zone of active cells which synthesize the tissue which eventually becomes the permanent articular cartilage of the joint. Deeper down in the cartilage a zone of ossification occurs. Just above this zone there exists a layer of replicating cells which produce cartilage for eventual endo-

chondral ossification of the epiphysis. At skeletal maturity, when no more cartilage becomes calcified, the osteochondral junction is well defined and is observable as a thin wavy blue line (the 'tide mark') on haematoxylin-eosin staining.

Human articular cartilage is colourless and semi-translucent during foetal life. It acquires an opacity during childhood and is usually white or yellow on attainment of adulthood. Chemical analyses of joint cartilage have revealed two phases in the life span of the tissue: an early 'developmental' phase in which the relative concentrations of the organic components show a progressive change, and a postdevelopmental or 'ageing' phase during which the biochemical composition of the tissue remains relatively static.

The early development phase of articular cartilage is associated with a progressive increase in collagen content and a progressive decline in hydration, total hexosamine, chondroitin sulphate levels, ash content, and sialoprotein levels (Kuhn and Leppelmann, 1957; Eichelberger, Akeson, and Roma, 1958; Campo and Tourtellotte, 1967; Bjelle, Antonopoulos, Engfeldt, and Hjertquist, 1972; Lust and others, 1972; Šimůnek and Muir, 1972a).

The changes in some of these properties are quite marked in pigs (Table II). The uronic acid (a measure of chondroitin sulphate) of pig articular cartilage fell from 7.6 per cent. of the dry weight of foetal tissue during the second half of pregnancy to 3.05 per cent. at 10 weeks of age (Šimůnek and Muir, 1972a). The collagen content increased from 19.6 to 38.2 per cent. and the water content decreased from 85.4 to 74.5 per cent. over the same period. Glucosamine to galactosamine molar ratio determinations of human and pig articular cartilage have demonstrated an increase in the relative proportion of keratan sulphate to chondroitin sulphate during this developmental phase (Kuhn and Leppelmann, 1958; Šimůnek and Muir, 1972a). The keratan sulphate fraction becomes more heterogeneous in size and sulphate content during this change in the glycosaminoglycan population (Bjelle and others, 1972).

It would appear that the collagen, later to become a relatively inert fibrillar network, undergoes considerable organization at fibril level during this developmental period. Silberberg, Silberberg, Vogel, and Wettstein (1961) monitored the fibrillar development in the joint cartilage of mice by electron microscopy.

Table II *Composition of pig knee-joint cartilage at five ages*

Age	Foetus	10 wks	25 wks	3 yrs	5 yrs
Dry weight (per cent. of wet wt)	14.6	25.5	29.9	34.7	36.3
Uronic acid (per cent. of dry wt)	7.60	3.05	3.04	3.10	2.85
Collagen (per cent. of dry wt)	19.6	38.2	49.0	52.7	54.6
Galactosamine/Glucosamine molar ratio	27	30	17	4	3.9

(From Šimůnek and Muir, 1972a).

Fibrils of diameter 10 nm. were present at birth but were not evident at 6 months of age. Collagen fibres of 30 nm. diameter and 60–70 nm. periodicity first appeared when the animal was 1 week old. Mature cartilage fibres of 70 nm. or more diameter and 60–70 nm. periodicity were evident 3 weeks after birth and formed the most abundant form of collagen in the older animals.

The cell density of articular cartilage, as measured by cell counts of fixed tissues or DNA estimations, shows a progressive decline with age in bovine (Wagoner, Rosenthal, and Bowie, 1941), rabbit (Barnett, Cochrane, and Palfrey, 1963; Mankin, 1964), canine (Lust and others, 1972), and guinea-pig joint tissue (Silberberg, Stamp, Lesker, and Hasler, 1970). In humans this decline in cellularity apparently ceases on maturity (Meachim and Collins, 1962; Stockwell, 1967a; Meachim, 1969). No mitotic activity has been observed in either the calcifying or superficial zones of normal tissue after definition of the osteochondral junction (Crelin, 1957; Mankin, 1963). The rate of protein synthesis in rabbit joint cartilage decreases up to the age of 6 months and remains constant thereafter (Mankin and Baron, 1965).

Little change occurs in the composition of articular cartilage after the animal is skeletally mature. In this respect articular cartilage differs from other cartilaginous tissues such as costal cartilage. No appreciable change with age has been noted in the water, collagen, total hexosamine, chondroitin sulphate, total nitrogen, sulphur, and ash content of the tissues (Anderson and others, 1964; Miles and Eichelberger, 1964; Linn and Sokoloff, 1965; Bollet and Nance, 1966; Maroudas and others, 1969; Šimůnek and Muir, 1972a).

The molar ratio of glucosamine to galactosamine of pig articular cartilage in animals aged 3 to 5 years was also constant (Šimůnek and Muir, 1972b). Furthermore, the chain length of chondroitin sulphate in human (Bollet and Nance, 1966) and pig (Šimůnek and Muir, 1972b) joint cartilage did not vary with the age of the subject. Miller and others (1969) demonstrated that the amino acid composition of human articular cartilage and the ease with which the collagen may be solubilized did not alter among individuals varying in age from 3 to 90 years. Lipid extractability from human joint cartilage is also apparently uninfluenced by age (Stockwell, 1967b).

The ease with which proteoglycans may be extracted from the articular cartilage is also unaffected by the age of the animal after attainment of maturity. Sequential extraction of articular cartilage from pigs varying in age from the foetal state to 5 years with 0.15M sodium acetate and 2.0M calcium chloride revealed that, whilst all the uronic acid of the foetal tissue was brought into solution, only 70 per cent. of the uronic acid from the joint cartilage of pigs of 25 weeks or older was solubilized. Although little varia-

tion in the relative proportions of the different sizes of the extracted proteoglycans was evident from tissue aged 25 weeks or older, the proportion of keratan sulphate relative to chondroitin sulphate increased progressively with age (Šimůnek and Muir, 1972a; Brandt and Muir, 1969b).

Proteoglycans display a rapid turnover, even in mature cartilage, and display a half-life of about 8 days (Mankin and Lippiello, 1969). Ageing has no appreciable effect on mean sulphate uptake in articular cartilage from the humeral head (Collins and Meachim, 1961). These considerations suggest that the chondrocytes of articular cartilage are very efficient in maintaining the constant composition of the extracellular matrix. The cells are presumably particularly sensitive to changes in the concentration of the organic constituents in their microenvironment.

The changes which take place in the articular cartilage during early life are apparently essential to the development of a tissue which, in later life, will meet the functional demand of the joint. The application of excessive stress or restriction on joint articulation during the maturation phase can seriously impair development of the cartilage. Pigs reared under conditions of intensive husbandry, where the mobility of the animal is restricted and the feeding regimen produces a greater body weight for their age, are predisposed to a joint lesion termed 'pig lameness' (Vaughan, 1971). Although the chemical composition of the articular cartilage of lame pigs is identical to that of normal animals, the extracted proteoglycans were of considerably smaller size than those from the normal cartilage (Šimůnek and Muir, 1972b).

Osteoarthrosis

Many types of joint injury and diverse pathogenic mechanisms can produce in the articular cartilage of all vertebrates a reaction pattern termed osteoarthrosis (synonym: osteoarthritis) (Gardner, 1965). The earliest morphological changes characterizing the disease include loss of cartilage elasticity and loss of cartilage flakes from the surface due to cleavage of the tissue along the horizontally-aligned fibrillar planes. Eventually, when the more randomly disposed collagen fibres in the tissue are exposed, deep vertical fissures arise. This process is termed fibrillation. In severe cases, total erosion of the cartilage with exposure of the underlying bone occurs. The incidence and severity of these lesions increase with age (Heine, 1926). The amount of abnormal physical stress, generated both by weight bearing and muscle pull, appears to be an important factor in the induction of cartilage breakdown. However, although the incidence of osteoarthrosis is normally greater in those weight-bearing sites which have been subjected to unusual stress (Bollet, 1969), typically non-weight-bearing joints are also affected. Furthermore, although cartilage erosion has been demonstrated to be

an age-correlated process, it is not a generalized pattern of ageing (Meachim, 1969).

Biochemical investigation into the nature of osteoarthritis has been confronted with two fundamental problems. The disease has never been defined in precise biochemical terms and the criteria of disease severity are therefore necessarily crude. The second problem, related to the first, is that of tissue sampling. The disease is considered to be focal and different metabolic events probably occur at any one time in different areas of an osteoarthrotic tissue. The selection of an appropriate control tissue to the pathological specimen under study is therefore difficult. The topographical variation in compressive stiffness (Kempson and others, 1969) and chemical composition of articular cartilage, and the possibility that certain effects of osteoarthritis may be more diffuse in the cartilage than others, suggest that an area of apparently 'normal' cartilage adjacent to an obviously pathological site may not be a valid control specimen. These considerations are presumably responsible, at least in part, for the lack of agreement in the published biochemical results in this field.

Early histochemical studies established a decrease in the normal metachromasia of articular cartilage in early degenerative disease of the joints. These observations were considered to indicate a loss of glycosaminoglycan from the tissue. Kuhn and Leppelmann (1957) and Anderson and others (1964) failed to find significant differences in the total nitrogen, sulphur, and hexosamine levels of mildly and severely affected osteoarthrotic articular cartilage specimens compared to normal tissues. Anderson and his co-workers, however, found considerable variation in the composition of different specimens, and this variation was apparently independent of the age, diagnosis, or anatomical site of the tissue. They suggested that degenerative disease is a focal process. The heterogeneous nature of osteoarthritis has been histologically demonstrated (Mankin, Dorfman, Lippiello, and Zarins, 1971), by the use of safranin-O, a stain which is both quantitative and specific for glycosaminoglycans (Rosenberg, 1971a).

Several investigators have detected a decrease in one or more of the glycosaminoglycans of articular cartilage in osteoarthritis. A decrease in total glycosaminoglycan (*i.e.* hexosamine) levels has been recorded by several groups of workers (Mankin and Lippiello, 1970, 1971; Matthews, 1953; Bjelle and others, 1972; Lust and Pronskey, 1972). This fall in polysaccharide content has been variously ascribed to a decrease in chondroitin sulphate (Bollet, Handy, and Sturgill, 1963; Bollet and Nance, 1966), keratan sulphate (Mankin and Lippiello, 1971; Benmaman, Ludoweig, and Anderson, 1969), or both (Bjelle and others, 1972; Kuhn and Leppelmann, 1958; Lust and Pronskey, 1972). An inverse correlation between the severity of the osteoarthrotic process and the glycos-

aminoglycan concentration has been noted (Bollet and others, 1963; Mankin and others, 1971; Mankin and Lippiello, 1970).

The decrease in chondroitin sulphate concentration observed by Bollet and Nance (1966) was associated with a decrease in the average chain length of the chondroitin sulphate as determined by reducing sugar end-group analyses. The degree of heterogeneity in the normal keratan sulphate population is apparently uninfluenced by the osteoarthrotic process (Bjelle and others, 1972). Mankin and Lippiello (1971) noted an increase in the degree of sulphation of the chondroitin 4 sulphate.

Collagen appears to play a secondary role in the disease. Osteoarthritis did not affect the hydroxyproline concentration of articular cartilage (Bollet and others, 1963; Anderson and others, 1964; Mankin and Lippiello, 1970) or the extractability of collagen from the tissue (Miller and others, 1969; McDevitt and Muir, unpublished). Furthermore, studies *in vitro* have failed to detect a collagenase in chondrocytes, leucocytes, and plasma which would degrade articular cartilage (Curtiss and Klein, 1963, 1965). However, the total disorganization of the collagen in the superficial layer (McCall, 1969) and the increased turnover of collagen in experimentally-induced osteoarthritis (Repo and Mitchell, 1971) suggest the participation of a collagen-degrading factor in the degenerative process. Collagen degradation could be purely mechanical (at least initially) or might involve a synovial collagenase (Harris, Cohen, and Krane, 1969).

Although early work (Meachim and Collins, 1962) has associated chondrocyte proliferation with osteoarthritis, more recent studies (Mankin and others, 1971) have demonstrated that the DNA concentration, an indicator of cell population, remains constant irrespective of the severity of the disease. The incorporation of thymidine- ^3H , an indicator of DNA synthesis, is increased during osteoarthritis (Mankin and Lippiello, 1970), and the degree of incorporation is proportional to the severity of the disease up to a certain grade of severity (Mankin and others, 1971). These latter workers detected a good inverse correlation between the DNA level and the ^3H -thymidine incorporation, suggesting that a decrease in cellularity was accompanied by a rise in mitotic activity.

The incorporation of $^{35}\text{SO}_4$, considered a fairly specific index of glycosaminoglycan synthesis, is increased in osteoarthritis (Bollet and Nance, 1966; Collins and Meachim, 1961; McElligott and Collins, 1960; Mankin and Lippiello, 1970; Repo and Mitchell, 1971). The $^{35}\text{SO}_4$ incorporation increased with the severity of the disease up to a certain pathological state. The degree of $^{35}\text{SO}_4$ incorporation appears to be dependent on the hexosamine concentration of the tissue, a decrease in the hexosamine

level being associated with an increased sulphate incorporation (Mankin and Lippiello, 1970; Mankin and others, 1971).

Little is known about the quality of the proteoglycan population in osteoarthrotic tissue. Preliminary results of ours suggest that the extractability of the proteoglycans from articular cartilage is markedly enhanced in canine joints which have naturally-acquired or experimentally-induced osteoarthritis, (Tables III and IV). Furthermore, the proteoglycans extracted from the osteoarthrotic tissue differed in chemical composition, as assessed by galactosamine/glucosamine molar ratios, from those extracted from the control tissues. The proteoglycans extracted from the abnormal cartilage were demonstrably different in hydrodynamic size by gel electrophoresis (McDevitt and Muir, 1971) from those extracted from normal tissue (McDevitt, Muir, and Pond, 1973). These results suggest the presence of a proteoglycan population in osteoarthrotic tissue which is more easily extracted and contains molecules which are smaller in size and contain a higher proportion of chondroitin sulphate chains relative to the number of keratan sulphate chains than the population of proteoglycans in normal cartilage. Whether these smaller macromolecules are degradative products of normal proteoglycans resulting from an increased catabolic rate, or represent newly formed but qualitatively imperfect proteoglycans, is as yet unclear. Organ culture studies

with chick limb bone rudiments suggest that the chondrocytes possess the capacity to resynthesize their matrix constituents after initial proteoglycan digestion by hyaluronidase (Fitton-Jackson, 1970). However, the newly synthesized proteoglycans were of smaller hydrodynamic size and had a lower uronic acid-protein ratio compared with controls (Hardingham, Fitton-Jackson, and Muir, 1972). The chondroitin sulphate chain lengths of these proteoglycans were normal. These considerations suggest that cartilage chondrocytes have a limited capacity for repair, in that the new proteoglycans apparently had fewer chondroitin sulphate chains on the core proteins than normal proteoglycans.

The pathogenesis of osteoarthritis is as yet unknown. The biochemical and metabolic phenomena described must be regarded as secondary effects of a disease which is already pathologically well advanced. Biomechanical studies suggest that depletion of the glycosaminoglycan fraction, with subsequent disruption of the collagen fibres in the superficial zone, is an early event in the disease process (Freeman, 1972). The creep modulus, a measure of the stiffness of a viscoelastic material, showed a close correlation with the total glycosaminoglycan content and a low correlation with the collagen content of human femoral head cartilage (Kempson, Muir, Swanson, and Freeman, 1970). Cartilage from normal femoral heads showed a systematic pattern of stiffness.

Table III Comparison of proteoglycans extracted with 2M-CaCl₂ from normal with those from pathological canine cartilage in natural osteoarthritis of the hip

Cartilage	Osteoarthrotic	Control
Uronic acid extracted (per cent. of total in tissue)	75.73	64.99
Protein extracted (mg./100 mg. dry wt of tissue)	5.238	5.112
Galactosamine/Glucosamine molar ratio		
(a) Starting tissue	6.52	4.75
(b) Extracted proteoglycans	9.30	5.12
(c) Cartilage residue after extraction	5.41	4.55

(From McDevitt, Muir, and Pond, 1973).
Control and affected dogs were 13 months old

Table IV Comparison of proteoglycans extracted with 2M-CaCl₂ from pathological cartilage in osteoarthritis experimentally-induced in one knee of a dog aged 2 years, with proteoglycans extracted from the other knee

Cartilage	Osteoarthrotic	Control
Uronic acid extracted (per cent. of total in tissue)	74.96	34.01
Protein extracted (mg./100 mg. dry wt)	8.830	6.550
Galactosamine/Glucosamine molar ratio		
(a) Starting tissue	4.67	3.40
(b) Extracted proteoglycans	5.82	3.38
(c) Cartilage residue after extraction	4.53	3.50

(From McDevitt, Muir, and Pond, 1973).
The osteoarthritis was induced by severing the anterior cruciate ligament and the dog was killed 5 months later.

Cartilage from visually normal areas of tissues showing degenerative changes from Grade 1 to Grade 4 (Byers, Contepomi, and Farkas, 1970) becomes progressively less stiff (*i.e.* softer) with the severity of the disease (Kempson and others, 1969). The osteoarthrotic process, although focal in its manifestations of fibrillation, showed a generalized decrease in stiffness. Furthermore, the change in creep modulus appears to precede the appearance of fibrillation. As the creep modulus is a function of the glycosaminoglycan concentration, it would appear that depletion of these constituents precedes, and may cause, the anatomical abnormality of fibrillation (Freeman, 1972).

The loss of glycosaminoglycans may result from cleavage of the proteoglycan 'core' protein by lysosomal cathepsin (Lucy, Dingle, and Fell, 1961; Dingle, 1962; Fell and Dingle, 1963; Fessel and Chrisman, 1964; Ali, 1964), particularly cathepsin D which degrades cartilage proteoglycans (Weston,

Barrett, and Dingle, 1969; Dingle, Barrett, and Weston, 1971). Hence the prime initiator of osteoarthrosis may be an agent, similar in action to vitamin A, which disrupts the surfaces of lysosomes. The chondrocytes apparently attempt to replenish the polysaccharide depletion by mitosis and by an increased rate of synthesis of protein and glycosaminoglycan. That they do not achieve their goal is demonstrated by the lower concentration of glycosaminoglycan which persists throughout the disease process. The rate of degradation of the proteoglycans always exceeds their rate of synthesis. The new proteoglycans synthesized by the cells appear to differ from those of normal mature cartilage in composition and, it seems, in molecular size. The higher galactosamine to glucosamine ratio observed by some workers suggests that the chondrocytes may synthesize a proteoglycan which is similar in composition to those found in immature cartilage.

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