Biosynthesis of Collagen and other Matrix Proteins by Articular Cartilage in Experimental Osteoarthrosis

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Osteoarthrosis was induced in one knee joint of dogs by an established surgical procedure. Changes in the articular cartilage in the biosynthesis of collagen and other proteins were sought by radiochemical labelling in vivo, with the following findings. (1) Collagen synthesis was stimulated in all cartilage surfaces of the experimental joints at 2, 8 and 24 weeks after surgery. Systemic labelling with [³H]proline showed that over 10 times more collagen was being deposited per dry weight of experimental cartilage compared with control cartilage in the unoperated knee. (2) Type-II collagen was the radiolabelled product in all samples of experimental cartilage ranging in quality from undamaged to overtly fibrillated, and was the only collagen detected chemically in the matrix of osteoarthrotic cartilage from either dog or human joints. (3) Hydroxylysine glycosylation was examined in the newly synthesized cartilage collagen by labelling dog joints in vivo with [³H]lysine. In experimental knees the new collagen was less glycosylated than in controls. However, no difference in glycosylation of the total collagen in the tissues was observed by chemical analysis. (4) Over half the protein-bound tritium was extracted by 4 M-guanidinium chloride from control cartilage labelled with [³H]proline, compared with one-quarter or less from experimental cartilage. Two-thirds of the extracted tritium separated in the upper fraction on density-gradient centrifugation in CsCl under associative conditions. Much of this ran with a single protein band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions. The identity of this protein was unknown, although it resembled serum albumin in mobility after disulphide-bond cleavage.

Articular cartilage is a specialized connective tissue that provides bearing surfaces in synovial joints. The cells are widely separated and lie within a stiff extracellular matrix consisting of a proteoglycan gel entrapped within a tight meshwork of collagen fibrils. The compressive stiffness of cartilage is due largely to the proteoglycans (Kempson et al., 1970), which retain water within the fibrous framework (Ogston, 1970).

At least five genetically distinct types of collagen molecule are known. The best characterized are the fibrillar species, types I, II and III (Miller, 1976). Type-IV collagen is in specialized basement membranes (Kefalides, 1975) and type-V collagen [or $\alpha A(\alpha B)_2$] is a minor component of many tissues

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(Burgeson et al., 1976; Chung et al., 1976). Type-II collagen is mainly located in hyaline cartilage and intervertebral discs (Eyre & Muir, 1975), and generally accounts for all the collagen in mammalian articular cartilage (Miller & Lunde, 1973; Eyre & Muir, 1975).

Osteoarthrosis is a widespread disease of vertebrate synovial joints in which progressive thinning of articular cartilage occurs at focal sites until, in severely advanced stages of the disease, the underlying subchondral bone is exposed (Lee et al., 1974; Howell et al., 1976; Peyron, 1979). Its pathogenesis is poorly understood, although mechanical and biochemical factors are both believed to be involved. Although osteoarthrosis can only be diagnosed at relatively advanced stages of the disease, the early phase may be investigated in experimentally induced osteoarthrosis in animals. In the experimental model employed in the present work the knee joint was

made unstable by cutting the anterior cruciate ligament (Pond & Nuki, 1973). The resulting changes in the bone (Gilbertson, 1975) and cartilage (McDevitt & Muir, 1976; McDevitt et al., 1977) were indistinguishable from those of natural osteoarthrosis. In both the experimental and natural disease the cartilage contained more water and a higher proportion of the total proteoglycans could be extracted, which suggested that the capacity of the fibrillar matrix to entrap the proteoglycans and to resist their swelling pressure was defective in the early stages of the disease (McDevitt & Muir, 1976; McDevitt, 1979).

It has been reported that, whereas type-II collagen is formed in normal articular cartilage, type-I collagen is synthesized in vitro by osteoarthrotic human articular cartilage (Nimni & Deshmukh, 1973) and by articular cartilage from inflamed rabbit joints (Deshmukh & Hemrick, 1976). Type-I collagen has also been detected immunochemically around clones of cells in human osteoarthrotic cartilage (Gay, 1978), and chondrocytes under certain conditions in culture produce a mixture of type-I collagen, type-I trimer and type-III collagen instead of type-II collagen (Layman et al., 1972; Schiltz et al., 1973; Mayne et al., 1975, 1976; Benya et al., 1977; Norby et al., 1977). On the basis of these observations, it is becoming accepted that the pathogenesis of osteoarthrosis features a switch in phenotype of the articular chondrocytes and a deposition of the wrong type of collagen in the matrix. The question whether articular chondrocytes continue to synthesize type-II collagen in vivo in osteoarthrosis, particularly in relatively advanced lesions, is examined in the present work. Cartilage from both early and well-developed lesions was compared with normal canine cartilage. Changes were sought in the biosynthesis of collagen and non-collagenous proteins. The type of collagen present in normal and osteoarthrotic human cartilage was also examined.

Experimental

Experimental animals

Five adult dogs were used. Three were female beagles, aged 6–8 years (12 kg in weight), that had been used solely for breeding. The other two were skeletally mature cross-bred dogs over 20 kg in weight. Experimental osteoarthrosis was induced in one knee by severing the anterior cruciate ligament by a stab incision, the other knee serving as a control (Pond & Nuki, 1973). The surgical procedure and the post-operative care of the animals have been described (Gilbertson, 1975; McDevitt et al., 1977). The dogs were killed at 2, 8 or 24 weeks after surgery as detailed in Table 1.

Radiochemical labelling

In four dogs the radioactive precursor was injected intra-articularly into both knees via the suprapatellar pouch; the fifth received the radioactive precursor into the cephalic vein (Table 1). In the first experiment dogs 1 and 2 received 1 ml of iso-osmotic saline (0.9% NaCl) containing 1 mCi of L-[5-³H]proline (0.5 Ci/mmol). In the second experiment dogs 3 and 4 received 5 mCi of L-[4,5-³H]lysine (15 Ci/mmol) plus 1 mCi of L-[5-³H]proline in 1 ml of iso-osmotic saline per knee. In the third experiment dog 5 received 10 mCi of L-[5-³H]proline intravenously. All dogs were killed by intravenous injection of sodium pentobarbitone 8 days after receiving the radioactive precursor(s) (McDevitt et al., 1977). Their joints were stored in sealed plastic bags at −20°C.

Dissection of cartilage

Joints were thawed at 4°C and the quality of the articular surface was graded by the Indian-ink procedure of Meachim (1972) (McDevitt & Muir, 1976): grade 1, 'intact' articular surface, with no uptake of Indian ink; grade 2, 'minimal fibrillation', with only light specks of Indian ink penetrating the articular surface; grade 3, 'overt fibrillation', with velvety sites of cartilage devoid of articular surface where Indian ink was retained as black patches. Cartilage from three sites of the tibia was sampled as follows (see McDevitt & Muir, 1976): medial plateau not covered by the medial meniscus, area A; remainder of the medial cartilage, area B; entire lateral plateau, area C. Subchondral bone and cartilage at the articular margins were rigorously excluded throughout. It has previously been observed in 18 dogs that the lesion always developed in area A of the tibia (McDevitt et al., 1977). Cartilage from the patella and the femur was similarly graded and sampled.

Human tissues

Fresh human articular cartilage was obtained from six osteoarthrotic knee joints and one femoral head identified at post-mortem in individuals that had no known generalized disorder of connective tissue and from an osteoarthrotic femoral head removed for total hip replacement. Cartilage surface quality was graded as above, and focal sites were sampled for analysis of collagen CNBr-cleavage peptides.

Extractions

The canine tissues were finely diced with a scalpel blade and immediately weighed. Proteoglycans were extracted by two different procedures. Cartilages of dogs 2, 3 and 5 were extracted at 4°C for 48 h in 2 ml of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing the following

proteinase inhibitors: 5 mM-benzamidine, 0.1 M-aminohexanoic acid and 10 mM-EDTA (Oegema *et al.*, 1975). The residual cartilage was washed for a few minutes with 1 ml of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, and the extract and washings were combined and dialysed overnight at 4°C against 7 vol. of 50 mM-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors. The second procedure was as follows. Cartilages of dog 1 were extracted sequentially with 0.15 M-sodium acetate buffer, pH 6.8, for 2 h and then with 4 M-guanidinium chloride/50 mM-EDTA/50 mM-sodium acetate buffer, pH 5.8, for 48 h at 4°C. The guanidinium chloride extract plus washings was dialysed against 7 vol. of 50 mM-EDTA/50 mM-sodium acetate buffer, pH 6.8. The 0.15 M-sodium acetate extract was filtered through a plug of glass-wool and assayed for hexuronate content and ^3H radioactivity. As these fractions contained less than 2% of the total uronate of the tissue, they were combined with the dialysed guanidinium chloride extracts before density-gradient centrifugation.

Density-gradient centrifugation

Proteins and proteoglycans were separated by equilibrium density-gradient centrifugation under associative conditions, essentially as described by Sajdera & Hascall (1969). The starting density was 1.5 g/ml, and a 10 × 10 ml titanium angle-head rotor was used at 95000 $g_{av.}$ at 10°C for 48 h. The tubes were then frozen in an ethanol/solid CO_2 bath and cut into three fractions comprising the bottom two-fifths, A1, the middle one-fifth, A2, and the top two-fifths, A3, by volume (nomenclature of Heinegård, 1972). The A1 and A2 fractions were dialysed against 50 mM-sodium acetate buffer, pH 6.8, overnight at 4°C, whereas the A3 fraction, including any undissolved material, was dialysed exhaustively against water at 4°C and freeze-dried.

Digestion with CNBr

Cartilage was digested with CNBr in 70% (v/v) formic acid as previously described (Eyre & Glimcher, 1973; Eyre & Muir, 1974). The entire sample was digested completely to give a cloudy solution, which was diluted 10-fold and then freeze-dried. Weighed portions of the dry material were hydrolysed for measurement of [^3H]proline and hydroxy-[^3H]proline (see below), and the remainder was used for analyses of collagen CNBr-cleavage peptides. This procedure avoided possible variations between individual pieces of cartilage and ensured that all analyses were representative of the entire sample.

Measurement of radioactivity in proline and hydroxyproline

Whole cartilage or CNBr digests of cartilage were hydrolysed in 6 M-HCl for 24 h at 108°C under N_2,

and the hydrolysate was evaporated to dryness. Hydroxyproline and proline were separated on a column (1 cm × 20 cm) of Dowex 50W (X8; 200–400 mesh), eluted at room temperature with 0.2 M-sodium citrate buffer, pH 3.25. In later work the short column (0.9 cm × 10 cm) of an amino acid analyser was used at 40°C with a similar buffer. Fractions of volume 2.2 ml were collected, and 2.0 ml portions were removed and mixed with 10 ml of detergent-based scintillation fluid (Insta-Gel; Packard Instrument Co.). Radioactivity was measured in a Packard liquid-scintillation spectrometer.

Column chromatography of collagen CNBr-cleavage peptides

Collagen CNBr-cleavage peptides were separated from sulphated proteoglycans and other acidic proteins and protein fragments on a column (1.5 cm × 10 cm) of DEAE-cellulose (Whatman DE-52), equilibrated at room temperature with 0.2 M-NaCl/30 mM-Tris/HCl buffer, pH 7.5. Fractions of volume 4 ml were collected, from each of which 0.4 ml was taken for determination of radioactivity. Collagen peptides were eluted in the void volume by this buffer and the more acidic components were then eluted with 1 M-NaCl/30 mM-Tris/HCl buffer, pH 7.5. Fractions containing collagen peptides were pooled, desalted by elution from a column (2.5 cm × 40 cm) of Bio-Gel P-2 (200–400 mesh) with 0.1 M-acetic acid, and freeze-dried.

The collagen CNBr-cleavage peptides were then fractionated by chromatography on a column (0.6 cm × 6 cm) of CM-cellulose (Whatman CM-52) as previously described (Eyre & Muir, 1974, 1975). Fractions of volume 2.2 ml were collected and 2 ml portions were taken for measurement of radioactivity. Where cartilage samples were labelled with both [^3H]lysine and [^3H]proline, all the effluent from the CM-cellulose column that contained radioactivity, apart from the void fraction, was pooled, desalted on the Bio-Gel P-2 column and freeze-dried.

Analysis of hydroxy[^3H]lysine and hydroxy[^3H]-lysine glycosides

Purified CNBr-cleavage peptides of collagen (<10 mg) from the dogs that had received [^3H]lysine were hydrolysed in 1 ml of 2 M-NaOH for 24 h at 110°C in polypropylene tubes sealed in glass (Eyre & Muir, 1975). The hydrolysate was diluted 5-fold, adjusted to about pH 2 with citric acid and finally diluted to 10 ml. The bulk of the sample was applied to the long column (0.9 cm × 60 cm) of an amino acid analyser and eluted with 0.35 M-sodium citrate buffer, pH 5.3, at 50°C. Fractions of volume 2.2 ml were collected and 2.0 ml portions were taken for scintillation spectrometry. A small portion of the sample was run under the same conditions but the

effluent was treated with ninhydrin to determine the total amounts of hydroxylysine and hydroxylysine glycosides present. Whole samples of human cartilage were similarly hydrolysed in alkali and analysed for hydroxylysine glycosides.

DEAE-cellulose chromatography of extracted proteins

Material extracted with 4 M-guanidinium chloride that separated at the top during CsCl centrifugation was fractionated on a column (0.9 cm × 10 cm) of DEAE-cellulose equilibrated at 42°C with 4 mM-Tris/HCl buffer, pH 8.5, flowing at 50 ml/h. The sample, which had been freeze-dried, was dissolved in and dialysed against a large volume of this buffer for several hours before it was applied to the column. Two consecutive salt gradients were applied: 0–0.15 M-NaCl in 150 ml of starting buffer, followed by 0.15–2.0 M-NaCl in a further 200 ml. Fractions of volume 4 ml were collected and 0.4 ml portions were taken for measurement of radioactivity. Fractions corresponding to selected peaks of radioactivity were pooled, and the material was desalted and hydrolysed for measurement of radioactivity in proline and hydroxyproline.

A comparable fraction prepared from the cartilage of a different dog that had received an intra-articular injection of [³⁵S]sulphate was chromatographed on the same column and radioactivity in the effluent fractions was measured.

Electrophoresis in sodium dodecyl sulphate/polyacrylamide gels

Two procedures were used, slab gels for analysis of collagen CNBr-cleavage peptides (Eyre et al., 1978), and cylindrical gels for analysis of extracted non-collagenous proteins where the radioactivity of serial slices was to be measured. Slab gels (1.5 mm thick) of 10% polyacrylamide were run according to the procedure of Neville & Glossman (1974), with Tris/borate buffers and 0.1% sodium dodecyl sulphate. Samples (50–100 μg) of CNBr-digested cartilage in starting buffer were denatured at 50°C for 5 min and placed in 1 cm-wide sample slots, and a current of 20 mA per gel was applied for 2–3 h. Proteins from the top fraction of density gradients were run on cylindrical gels in 0.1% sodium dodecyl sulphate/phosphate buffer (Furthmayr & Timpl, 1971) as described by Eyre & Muir (1975). Each sample was run with and without 2-mercaptoethanol (10 mM) in the buffer. Unstained gels were sliced serially into 3 mm-wide pieces, and the radioactivity was measured after each slice had been digested with 0.5 ml of 90% (w/v) H_2O_2 for 24 h at room temperature in a sealed vial and then 10 ml of scintillation fluid had been added. Cylindrical gels that were stained with Coomassie Brilliant Blue R were scanned in a densitometer (Eyre & Muir, 1975).

Amino acid analysis

The amino acid composition of the mixture of collagen peptides purified by CM-cellulose chromatography was determined after hydrolysis in 6 M-HCl for 24 h at 108°C in tubes partially evacuated under N_2. A single-column instrument (Locarte Co., London, U.K.) was used as previously described (Eyre & Muir, 1975).

Results

Grading of osteoarthrotic lesions

The quality of the articular surfaces of the knee-joint cartilage of four dogs killed 2, 8 and 24 weeks after surgery is shown in Table 1. Total loss of

Table 1. *Labelling procedure with experimental dogs and their grades of osteoarthrotic lesions*
Key: grade 1, normal intact articular surface; grade 2, minimal damage to surface; grade 3, overt fibrillation and loss of articular surface. The grading procedure (Meachim, 1972) and key to sites of tibial cartilage are described in the Experimental section. All control cartilage was grade 1.

Dog	Duration of experimental osteoarthrosis (weeks)	Radiochemical label	Grades of articular surfaces in experimental joints				
			Tibia				
			A	B	C	Femur	Patella
1. Cross-breed	8	Intra-articular [³H]proline	3	2	2	1	1 + 2
2. Cross-breed	24	Intra-articular [³H]proline	3	2	2	2	—
3. Beagle	2	Intra-articular [³H]proline + [³H]lysine				Not graded	
4. Beagle	8	Intra-articular [³H]proline + [³H]lysine	3	2	2	—	1
5. Beagle	2	Intravenous [³H]proline	2	1	1	1	1

the articular surface (grade 3) was evident in tibial area A 8 weeks after surgery compared with mild surface roughening (grade 2) in the adjacent cartilage (tibia area B) and in the lateral plateau (tibia area C). The progressive deterioration of the articular surface of tibial area A is consistent with previous findings in 18 dogs killed 1–48 weeks after surgery (McDevitt et al., 1977). It should be noted that the Indian-ink grading system of Meachim (1972) merely assesses the quality of the articular surface, and that progressive development of the lesions throughout the entire depth of the cartilage in area A of the tibia was evident in histological sections (McDevitt & Muir, 1976).

Relative incorporation of ³H label into collagen and non-collagenous proteins

Table 2 compares the distribution of the ³H label in the associative density gradient and the insoluble cartilage residue of control and operated knees injected with [³H]proline alone. More than one-half the radioactivity was extracted from the control cartilages compared with only about one-fifth from the pathological specimens. In the animals that had received intra-articular injections of both [³H]proline and [³H]lysine, 80% of the total radioactivity was extracted from control cartilage, compared with 60% from the cartilage of operated knees.

Associative equilibrium density-gradient centrifugation separated the ³H radioactivity of the extracts into a high-density fraction (A1) and a low-density (A3) fraction. Over 80% of the ³H radioactivity was concentrated in the low-density A3 fraction, where collagen and other proteins normally separate, and 8–18% was found in the high-density A1 fraction, where over 90% of the proteoglycans separate. Little difference was noted between pathological and control specimens in the distribution of ³H radioactivity in the associative gradient.

No more than 10% of the total hydroxy[³H]-proline was extracted from either operated or control cartilage specimens. Essentially all the labelled collagen remained in the guanidine-insoluble cartilage residues.

Table 3 shows how ³H label was distributed in the cartilage residue between collagen and non-collagenous proteins, as assessed by the radioactivity of proline and hydroxyproline. Radioactive proline and hydroxyproline separated completely under the chromatographic conditions used, and together accounted for at least 98% of the radioactivity applied to the column. The ratio of radioactivity in collagen to that in non-collagenous proteins was several times greater in all areas of the cartilage of the operated joints compared with the corresponding sites of the control joints. This difference must reflect changes in the relative rates of synthesis and degradation of proteins labelled from a common pool of [³H]proline. In cartilage residues from operated knees of dogs killed 2 (dog 3), 8 (dogs 1 and 4) and 24 (dog 2) weeks after surgery, 80% of the total radioactivity was in collagen compared with 20% in controls (assuming that in collagen the proline/hydroxyproline molar ratio is 1.2:1). In tibial area A of the operated joint, the site of the earliest and most severe lesions, almost all the label was in collagen 24 weeks after surgery. The proportion of label in collagen in the control cartilage was also somewhat increased after this time.

Systemic labelling

The results from intra-articular injection of [³H]proline suggested that the experimental cartilage synthesized more collagen per dry weight of

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Table 2. *Extraction and distribution in associative density gradients of ³H radioactivity from cartilage of experimental and control joints*

The dog (dog 2) was killed 24 weeks after surgery and [³H]proline was injected into each knee joint 8 days before death. The 4 ml of guanidinium chloride extract was dialysed and fractionated by associative density-gradient centrifugation into three fractions A1 ($\rho = 1.62$ g/ml); A2 ($\rho = 1.50$ g/ml); A3 ($\rho = 1.38$ g/ml). All the ³H radioactivity in extract and residue was shown to be in macromolecules.

	Control joint				Experimental joint			
	Tibia				Tibia			
	A	B	C	Femur	A	B	C	Femur
(a) % of total ³H extracted	61	65	62	57	20	15	36	25
(b) % distribution of extracted ³H in gradient								
A1	19	8	14	9	17	18	16	8
A2	2	4	3	3	2	1	0	4
A3	79	88	83	88	81	81	84	88

Table 3. *Incorporation of [³H]proline into collagen by articular cartilage of control and experimental knee joints at 2, 8 and 24 weeks after surgery*

Measurements were made on tissue slices after extraction of soluble proteins and proteoglycans by 4 M-guanidinium chloride. No more than 10% of the total hydroxy[³H]proline in control or experimental tissues was extracted by 4 M-guanidinium chloride. Tissue was hydrolysed and radioactivity in hydroxy[³H]proline and [³H]proline was measured after their separation by ion-exchange chromatography.

[³H]Hyp as a proportion of total protein-bound ³H radioactivity (%)*

Dog no.	Time after surgery (weeks)	Control joint					Experimental joint				
		Tibia			Femur	Patella	Tibia			Femur	Patella
		A	B	C			A	B	C		
1	2		Pooled 5.9		7.7	3.2		—		32.7	22.3
2	8		Pooled 3.7		7.9	3.6		Pooled 25.6		36.4	37.1
4	8	6.8	5.0	8.6	—	8.6	36.4	38.2	38.2	—	40.0
5	24	10.9	9.1	11.4	25.4	18.6	45.0	39.5	25.0	34.5	45.0

$$* \frac{[^3H]Hyp}{[^3H]Hyp + [^3H]Pro} \times 100$$

Table 4. *Incorporation of [³H]proline into collagen by control and experimental cartilages of a systemically labelled adult dog*

[³H]Proline (10 mCi) was injected intravenously 6 days after operation and 8 days before death. Measurements were made on hydrolysates of whole unextracted cartilage.

	Total ³H radioactivity (c.p.m./mg of dry cartilage)		Proportion of ³H radioactivity in [³H]Hyp (%)		Experimental/control ratio of [³H]Hyp/mg dry wt.
	Control	Experimental	Control	Experimental	
Articular cartilage					
Tibia { A	40	117	1.5	6.1	11.9
B	37	104	2.6	10.2	11.0
C	48	104	3.1	10.8	16.4
Femur	39	105	3.1	15.7	13.6
Fibrocartilage					
Semilunar meniscus	44	161	4.6	14.1	11.2
Intervertebral disc					
Annulus fibrosus	—	8	—	—*	
Nucleus pulposus	—	8	—	—*	
Laryngeal cartilage (thyroid plate)	—	12	—	—*	

* Total radioactivity too low to enable measurement of hydroxy[³H]proline.

tissue than did control cartilage. However, since the site of intra-articular injection of [³H]proline was unlikely to be uniform, reliable comparisons between different knees and areas of cartilage were not possible. One animal therefore was given [³H]proline intravenously to compare relative incorporations in the two knees. The entire cartilage was used rather than the residue after extraction with guanidinium chloride. Other cartilaginous tissues were also examined. Table 4 shows that the proportion of ³H radioactivity in collagen was about 4 times greater in all cartilage sites of the operated joint compared with their corresponding sites in the control knee. Furthermore, the absolute radioactivity in collagen, expressed as ³H radioactivity in hydroxyproline per dry weight of tissue, was more than 10 times higher in the cartilages of the operated joint than in corresponding controls. The radioactivity of the total protein (collagen plus non-collagenous protein) was only 2–3 times greater.

Incorporation into collagen by the load-bearing surfaces of the menisci of operated joints was also greatly elevated compared with controls. The other specialized cartilages, laryngeal cartilage (thyroid plate) and intervertebral disc, incorporated much less radioactivity into protein than did the cartilages of the synovial joint.

Type of collagen

The difference between control and diseased articular cartilages in their relative labelling of collagen and non-collagenous protein was confirmed on fractionation of CNBr digests of tissue on DEAE-cellulose (Fig. 1). Much more of the ^{3}H radioactivity in the diseased tissue was eluted with the voided fraction of collagen peptides. Analysis confirmed that all the hydroxy[^{3}H]proline was eluted in the void and only [^{3}H]proline in the later peak.

The increased incorporation of [^{3}H]proline into collagen at 2, 8 and 24 weeks after surgery went exclusively into type-II collagen, as assessed by the elution from CM-cellulose of CNBr-derived collagen peptides. The quality of the articular surface varied from grade 1 to grade 3. Fig. 2 shows the CM-cellulose elution profiles at 8 weeks; similar profiles were obtained at 2 and 24 weeks. The elution profiles of ^{3}H radioactivity seemed to follow the absorbance profiles of the type-II-collagen peptides even more closely for the analyses of experimental cartilage (Figs. 2b and 2c) than of control cartilage (Fig. 2a). Note that ^{3}H radioactivity is much higher in the Fig. 2(b) profile compared with the Fig. 2(a) profile, despite the smaller load of cartilage. This is
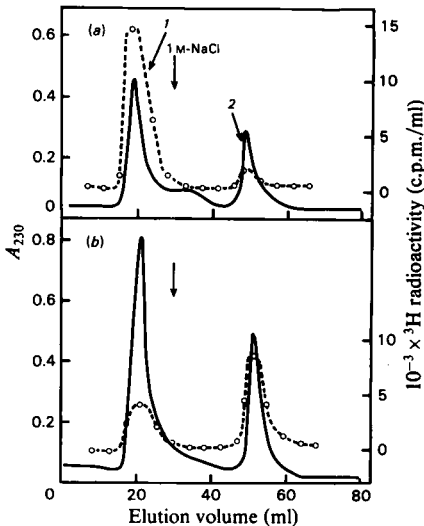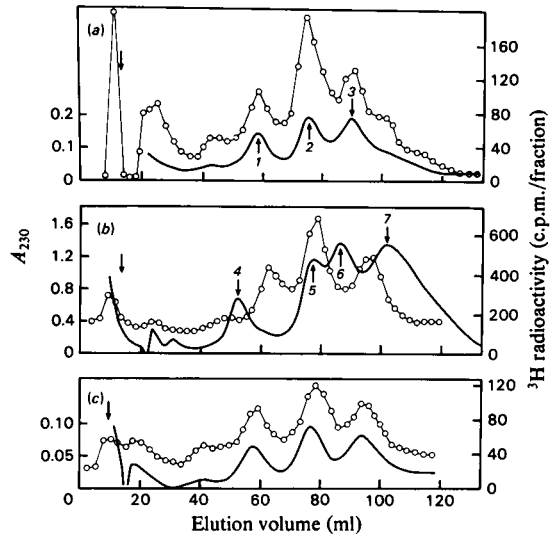

Fig. 2. *Chromatography on CM-cellulose of collagen CNBr-cleavage peptides from articular cartilage of control and experimental knee joints*

Tissue came from a single dog (dog 1) 8 weeks after induction of osteoarthrosis and 8 days after injection of each knee with [^{3}H]proline. The analyses are of peptides purified by DEAE-cellulose chromatography (as in Fig. 1) from 15 mg dry wt. of cartilage from control patella (*a*), 5 mg from experimental patella with a grade-1–2 osteoarthrotic lesion (*b*) and 6 mg from area A on the experimental tibia with a grade-3 lesion (*c*). An excess of 25 mg of CNBr-cleavage peptides from type-I collagen of non-radioactive dog bone was mixed with sample (*b*) to show their distinctly different elution profile compared with the radioactive type-II-collagen peptides. The column (0.9 cm × 6 cm) was equilibrated at 42°C with 20 mM-sodium citrate buffer, pH 3.6, and peptides were eluted by a linear gradient of 0.02–0.15 M-NaCl in 150 ml of buffer, begun at the arrows. ———, A_{230}; O—O, ^{3}H radioactivity. Key to peaks: *1*, peptide α_1(II)CB8; *2*, peptides α_1(II)CB9,7 and α_1(II)CB10; *3*, peptides α_1(II)CB11 and α_1(II)CB12; *4* peptide α_1(I)CB3; *5* peptide α_1(I)CB7; *6*, peptide α_1(I)CB8; *7*, peptides α_2CB3, α_2CB5 and α_2CB3,5.

Fig. 1. *Chromatography on DEAE-cellulose of CNBr digests of dog articular cartilage labelled in vivo with [^{3}H]proline*

The samples came from tibial area A of the experimental (*a*) and control (*b*) knees of dog 2 (see Table 1). The CNBr-cleavage peptides of collagen are eluted unretarded by the 0.2 M-NaCl/30 mM-Tris/HCl buffer, pH 7.5. At the arrow, 1.0 M-NaCl/30 mM-Tris/HCl buffer, pH 7.5, was applied to elute the acidic proteoglycan fragments. ———, A_{230}; O--O. ^{3}H radioactivity of the fractions. Key to peaks: *1*, collagen CNBr-cleavage peptides; *2*, proteoglycan fragments.

because of the 10-fold higher specific radioactivity of collagen in the experimental cartilage compared with control. On re-running another sample of the material used for Fig. 2(b) without carrier type-I-collagen peptides, the elution profile of ^{3}H radioactivity was identical, and it followed the underlying profile of absorbance of the endogenous type-II-collagen peptides obscured in the Fig. 2(b) profile (not shown). In general, patellar cartilage was more actively labelled than was tibial cartilage in joints injected intra-articularly with radioactive precursor.

The amino acid composition of the pooled mixture of CNBr-cleavage peptides recovered from the CM-cellulose column was typical of pure type-II collagen (Table 5), and analyses of preparations from control and experimental knees were indistinguishable.

Electrophoresis of CNBr-derived collagen peptides in sodium dodecyl sulphate/polyacrylamide gels

Samples of cartilage from experimentally induced canine osteoarthrosis and natural human osteoarthrosis were examined. No differences were detected in the electrophoretic patterns of CNBr-derived collagen peptides of control and osteoarthrotic cartilage (Fig. 3). The three human samples came from a single femoral head. The sample from the severest lesion, grade 4, came from an area near the fovea that was eroded to bone in parts but nevertheless appeared to be a remnant of original articular cartilage. Type-II collagen alone was detected in all samples, even the grade-4 lesion. Were any type-I collagen present it would not have accounted for more than 2% of the total collagen in any of the samples. Control experiments in which known mixtures of type-I and type-II collagens were run established this.

Glycosylation of hydroxylysine

It was found that cartilage could not simply be hydrolysed and analysed directly to measure radioactivity in hydroxylysine and hydroxylysine glycosides. The proportion of ^{3}H radioactivity in hydroxylysine in whole tissue was low, and other peaks of radioactivity, probably uncleaved peptides containing [^{3}H]lysine, interfered with the quantitative determination of the small peaks of glucosylgalactosylhydroxylysine, galactosylhydroxylysine and hydroxylysine. Accurate measurements could be made on the purified CNBr-cleavage peptides of collagen, however.

Fig. 4 shows an elution profile for such a sample. Table 6 compares the results for control and experimental tissues. The significant finding was that the extent of hydroxylysine glycosylation differed in the newly synthesized collagen measured radiochemically compared with that of the total collagen in the same sample measured chemically. This was

Table 5. *Amino acid composition of type-II collagen for dog articular cartilage*

The complete mixture of CNBr-cleavage peptides isolated by CM-cellulose chromatography, and representing essentially the whole molecule, was analysed.

	Amino acid composition (residues/1000 amino acid residues)	
	Human*	Canine
3-Hyp	2	1
4-Hyp	99	108
Asp	42	43
Thr	20	20
Ser	27	27
Glu	90	93
Pro	122	113
Gly	333	334
Ala	100	97
Val	18	16
Met†	9	7
Ile	9	10
Leu	26	25
Tyr	1	2
Phe	13	13
Hyl	14	20‡ { Glc-Gal-Hyl 6 / Gal-Hyl 6 / Hyl 8
Lys	22	16
His	2	4
Arg	51	51

* The mixture of CNBr-cleavage peptides from type-II collagen of human articular cartilage calculated from Miller & Lunde (1973).

† Measured as homoserine plus homoserine lactone.

‡ Four individual preparations from tibial and femoral cartilages of control and experimental knees of a single dog taken 8 weeks after surgery all gave 20 residues of hydroxylysine/1000 residues.

true for both control and experimental cartilages. However, in cartilage from operated joints the newly synthesized collagen was less glycosylated than the total collagen, whereas in control cartilage the newly synthesized collagen was more glycosylated than the total collagen. For example, in the dog at 8 weeks after surgery chemical analysis showed that hydroxylysine glycosylation was constant at 62% ($\pm$3%) for cartilages of both knees, but by radioactivity only 53–56% was glycosylated in the operated knee whereas 70% was glycosylated in the control. These differences were also evident in the dog at 2 weeks after surgery. Moreover, there were differences, particularly in controls, between total collagen and newly formed collagen in the molar proportions of the three forms of hydroxylysine, i.e. unsubstituted hydroxylysine, galactosylhydroxylysine and glucosylgalactosylhydroxylysine. The difference in degree of glycosylation of the total
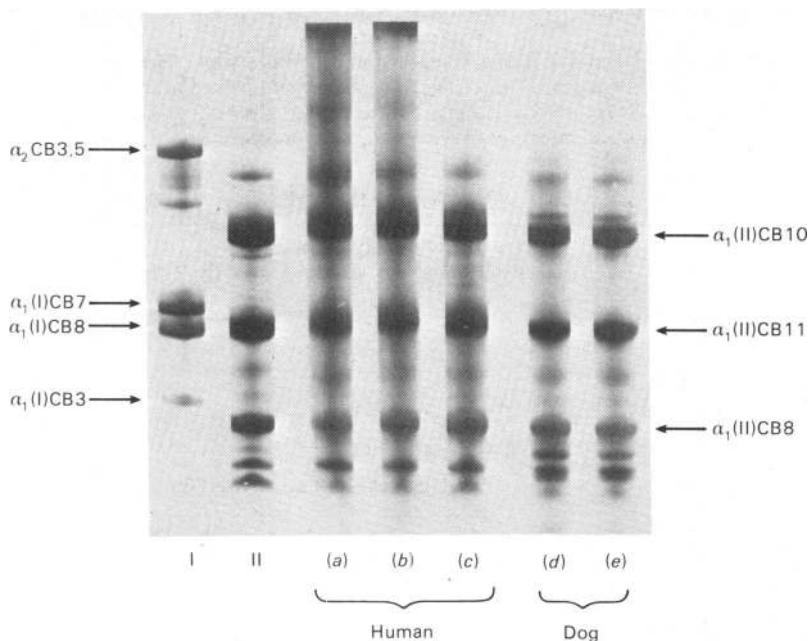
Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of CNBr digests of osteoarthrotic articular cartilage from man and dog*

Slabs (20 cm × 20 cm) of polyacrylamide gel (10%), 1.5 mm thick, were used. About 40 μg of digested tissue was run in each track. Standard digests of type-I collagen of pig fibrocartilaginous meniscus (track 1) and type-II collagen of pig laryngeal cartilage (track 2) were run (Eyre & Muir, 1975). The human tissue was sampled from a femoral head removed at post-mortem (46-year-old female), in which osteoarthrotic lesions were obvious. The samples came from sites that were graded according to Meachim's (1972) scale as grade 1, intact surface (*a*), grade 3, overt fibrillation (*b*), and grade 4, partial erosion to bone (*c*). For (*c*), care was taken to avoid sampling exposed bone or replacement fibrocartilage. Graded specimens from knee joints and from an osteoarthrotic femoral head removed at surgery gave similar results. The samples of dog cartilage came from area B on the tibia of the control knee (*d*) and area B on the tibia (grade-2 lesion) of the experimental knee (*e*) of an animal 24 weeks after the anterior cruciate ligament had been cut (dog 2). The major CNBr-cleavage peptides of type-I and type-II collagens are identified.
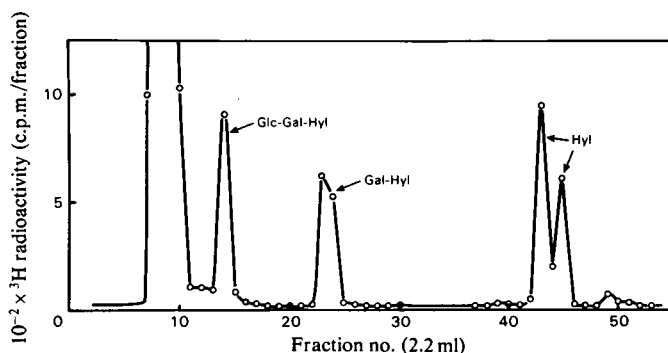

Fig. 4. *Ion-exchange column chromatography of hydroxylysine and the two hydroxylysine glycoside derivatives from the collagen of dog articular cartilage labelled in vivo with [³H]-lysine*

The long column (0.9 cm × 60 cm) of the amino acid analyser was eluted with 0.35 M-sodium citrate buffer, pH 5.28, at 50°C. Collagen CNBr-cleavage peptides isolated by CM-cellulose chromatography (Fig. 2) were pooled, desalted and hydrolysed in 2 M-NaOH before analysis. The elution profile shown was derived from articular cartilage of an operated knee joint injected with [³H]lysine 6 days after the anterior cruciate ligament had been cut (dog 3).

Table 6. *Radiochemical measurements of hydroxylysine glycosylation in newly synthesized collagen of dog articular cartilage*

Results are for two dogs (dogs 3 and 4 in Table 1) labelled in each knee with [³H]lysine 8 days before being killed at 2 and 8 weeks after surgery. Collagen CNBr-cleavage peptides were pooled after CM-cellulose chromatography (Fig. 2) and hydrolysed in alkali. Hydroxylysine and hydroxylysine glycosides were separated on the amino acid analyser and their ³H radioactivity and molar ratios were measured on separate portions of the hydrolysate.

| | | Dog 1 (2 weeks after surgery) | | Dog 2 (8 weeks after surgery) | |
		By ³H label	By chemical analysis	By ³H label	By chemical analysis
		Hyl residues glycosylated (%)*			
Control joint	Tibia†	82	—	—	65
	Femur	84	72	70	61
Operated joint	Tibia†	63	—	56	61
	Femur	65	—	53	62
		Glc-Gal-Hyl/hexose-free Hyl (molar ratio)			
Control joint	Tibia†	4.2	—	—	0.95
	Femur	2.9	1.3	1.63	0.70
Operated joint	Tibia†	1.0	—	0.65	0.81
	Femur	1.1	—	0.47	0.75

* Calculated from

$$\frac{\text{Glc-Gal-Hyl} + \text{Gal-Hyl} \times 100}{\text{Glc-Gal-Hyl} + \text{Gal-Hyl} + \text{non-glycosylated Hyl}}$$

with the use of mol or c.p.m. for Glc-Gal-Hyl, Gal-Hyl and non-glycosylated Hyl.
† Areas A, B and C were pooled.

Table 7. *Glycosylation of hydroxylysine in collagen of normal and osteoarthrotic human articular cartilage*

Whole tissue was hydrolysed in alkali, and the molar yields of hydroxylysine and the two hydroxylysine glycosides were measured directly on the amino acid analyser.

| | Normal* | | Osteoarthrotic† | | |
| | | | 1 | 2 | |
	16 years old	68 years old	Femur	Femur	Tibia
% of Hyl glycosylated	63	68	59	70	70
Glc-Gal-Hyl/Gal-Hyl	0.84	0.61	1.14	0.90	1.00
Glc-Gal-Hyl/non-glycosylated Hyl	0.77	0.83	0.76	1.14	1.16

* From Eyre & Muir (1977).
† Two different subjects. All samples came from grade-3 lesions in the knee joint.

collagen between the two dogs (62 and 70%) is not unreasonable. Human cartilage from different individuals also showed considerable variation in this respect (Table 7; Eyre & Muir, 1977).

Density-gradient centrifugation of non-collagenous proteins

Proteins and proteoglycans extracted from cartilage labelled with [³H]proline and [³H]lysine were fractionated by CsCl-density-gradient centrifugation. One dog was killed 2 weeks (dog 3) and the other 8 weeks (dog 4) after surgery. Three-quarters of the radioactivity extracted from both control and experimental cartilages separated at the top of the gradient, and one-quarter at the bottom. Of the radioactivity in the top fraction incorporated from [³H]proline, collagen accounted for $17 \pm 6\%$ for experimental and $3.3 \pm 0.6\%$ for control samples.

Each mean value and standard error was derived from five separate cartilage specimens from the two dogs.

DEAE-cellulose chromatography of non-collagenous proteins

The proteins in the top fraction of the density gradients were fractionated by chromatography on DEAE-cellulose (Fig. 5). The elution position of sulphated proteoglycans was established by running a similar preparation from a dog given [^{35}S]sulphate by intra-articular injection (Fig. 5a). Only a small proportion of the total extracted proteoglycans separated at the top of the associative gradient. Being of low buoyant density, these would be rich in protein.

In the material from the control cartilage more than one-half the eluted radioactivity was associated with proteoglycan, with a smaller amount in a peak eluted immediately before it (Fig. 5b). No hydroxy[^{3}H]proline could be detected in the material of either peak. In samples of osteoarthrotic cartilage there was consistently more radioactivity in the material that eluted before proteoglycan, and all the hydroxy[^{3}H]proline was recovered in the small peak that was eluted in the position of a standard preparation of α_1(II)-chain (Fig. 5). The small amount of radioactivity in the void peak was attributable to free [^{3}H]lysine. Any α_1(I)- or α_2-chains from type-I collagen in the extract should have been eluted in this void fraction.

Less than one-third of the radioactivity applied to the DEAE-cellulose column was recovered from either control or experimental samples, and consistently less of the radioactivity of control samples was eluted.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of non-collagenous proteins

Much of the radioactivity in the top density-gradient fraction of material extracted from control and osteoarthrotic cartilage was accounted for by a single protein band prominent on electrophoresis in sodium dodecyl sulphate/polyacrylamide gels after reduction with mercaptoethanol (Figs. 6 and 7).

Before reduction with mercaptoethanol, protein penetrating the gel was distributed in a broad zone (Fig. 6b) ranging in molecular weight from 40000 to 70000, with the most intense stain at 60000 mol.wt. Molecular weights were calibrated in the absence of mercaptoethanol by using bovine serum albumin, ovalbumin, pepsin, concanavalin A, chymotrypsin, ribonuclease and link protein from pig cartilage as standards. [The last protein gives a single band of about 45000 mol.wt. (D. R. Eyre, unpublished work).] When the gels were sliced most of the radioactivity was found at the origin and no other discrete bands were seen (Fig. 7).

On reduction with mercaptoethanol, however, there appeared a major protein band with apparent mol.wt. 110000, and the material at the origin disappeared (Fig. 6). When serum albumin was run
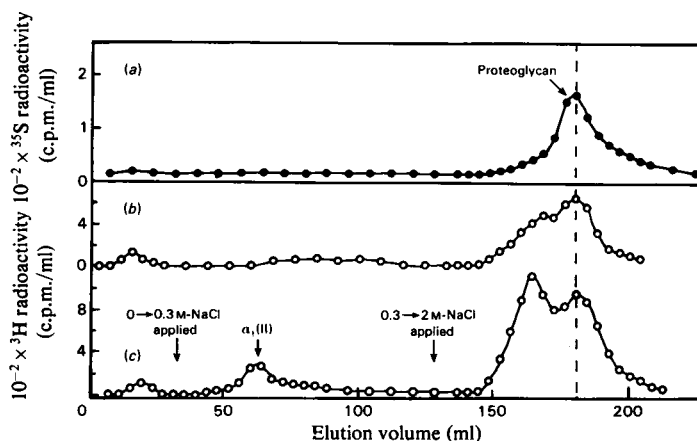

Fig. 5. *DEAE-cellulose chromatography of the low-density fraction of proteoglycans and proteins extracted from radioisotopically labelled dog articular cartilage*

The samples were prepared from cartilage of a control joint labelled *in vivo* by intra-articular injection of [^{35}S]sulphate (a) and from femoral cartilage of the control (b) and experimental (c) joints of an animal (dog 3) injected intra-articularly with [^{3}H]lysine plus [^{3}H]proline 6 days after induction of osteoarthrosis. Material recovered in the top two-fifths after density-gradient centrifugation of a 4 M-guanidinium chloride extract of the cartilage was chromatographed. The elution position of the α_1(II)-chain was calibrated in a separate run by using an α_1(II) preparation from pig laryngeal cartilage.
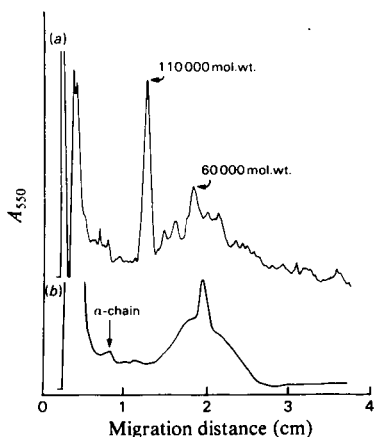
Fig. 6. *Sodium dodecyl sulphate/polyacrylamide-gel*
(7.5%) electrophoresis of the low-density fraction of
proteins extracted from dog articular cartilage

Samples (20–40μg) of the material recovered in the
top fraction after density-gradient centrifugation of a
4 M-guanidinium chloride extract of pooled tibial
cartilage from a control joint (dog 4) were run with
(a) and without (b) 10mM-2-mercaptoethanol. Den-
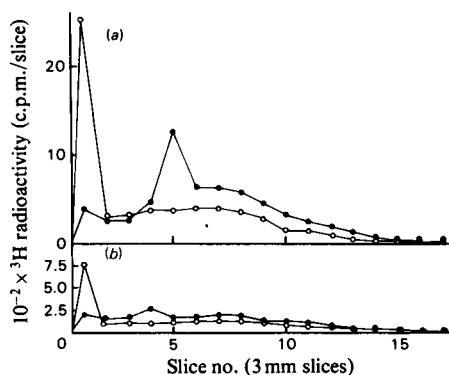sitometric profiles of protein bands stained with
Coomassie Blue are shown.

as the unfolded chain after treatment with mer-
captoethanol it had a similar mobility. In control
samples, about half the total protein and radio-
activity was in this component (Figs. 6 and 7).
Staining intensity suggested there was more in
extracts of control cartilage compared with experi-
mental (not shown).

When cut out and hydrolysed only [³H]proline
and [³H]lysine but no hydroxy[³H]proline or
hydroxy[³H]lysine were detected in the band. None
of this protein was detected either by staining or
by radioactivity on electrophoresis of radioactive
material recovered from DEAE-cellulose chromato-
graphy (Fig. 5). The fraction eluted from DEAE-
cellulose just before proteoglycan gave a broad
smear of radioactivity similar to the protein distri-
bution of unreduced samples seen in Fig. 6(b), but
the stained gel showed essentially the single protein
band of about 60000mol.wt.

Electrophoresis of the top density-gradient frac-
tion prepared from the cartilage of a dog injected
with [³⁵S]sulphate showed no radioactivity asso-
ciated with the 110000-mol.wt. band. Radioactivity
profiles were identical before and after reduction
with mercaptoethanol, with little of the ³⁵S-labelled
material penetrating the gel (not shown).

Fig. 7. *Sodium dodecyl sulphate/polyacrylamide-*
gel (7.5%) electrophoresis of the low-density frac-
tion of proteins extracted from radioisotopically
labelled dog articular cartilage

Samples (40–80μg) of material that were recovered
in the top fraction on density-gradient centrifu-
gation of 4 M-guanidinium chloride extracts of tibial
cartilages from control (a) and experimental (b) knee
joints were run. The dog (dog 4) was labelled
intra-articularly with [³H]proline plus [³H]lysine in
both knees 8 weeks after induction of osteoarth-
rosis. (The control sample was the preparation used
for the analyses shown in Fig. 6.) ○, No reduction
with 2-mercaptoethanol; ●, after reduction with
2-mercaptoethanol.

Discussion

In the experimental model of osteoarthrosis used
in the present work, changes in the quantity, quality
and organization of the proteoglycans occur before
damage to the articular surface is demonstrable by
histology (McDevitt & Muir, 1976; McDevitt *et al.*,
1977). The higher water content of the articular
cartilage (Bollet, 1967), suggesting a decrease in the
tensile strength of the collagen network (Maroudas,
1973), was evident in the very early stages of the
experimental disease. This change apparently per-
sists throughout the disease process, as it is a
well-documented feature of cartilage in advanced
osteoarthrosis (McDevitt, 1973).

Type of collagen

The pool of newly synthesized collagen in the
matrix of both control and osteoarthrotic cartilage
was predominantly type II, which is the normal
collagen type of the cartilage matrix (Miller &
Lunde, 1973; Eyre & Muir, 1975). From analysis of
CNBr-cleavage peptides, no type-I collagen could be
demonstrated in the experimental osteoarthrotic
cartilage, either chemically or by ³H-labelling, even
in specimens in which the specific radioactivity of
hydroxyproline was increased over 10-fold and the
lesion was grade 3. The small amount of labelled
collagen extracted by guanidinium chloride also was
identified as type II. Moreover, in specimens of

human osteoarthrotic cartilage with grade-4 lesions, again only type-II collagen could be detected, care having been taken to sample the original articular cartilage and omit regions that had been resurfaced with fibrocartilage, which is known to contain type-I collagen (Eyre, 1975; Eyre & Muir, 1975; Furukawa *et al.*, 1980). Thus the present findings do not support the view that in early osteoarthrosis chondrocytes in articular cartilage synthesize and deposit in the matrix type-I collagen in place of type-II collagen.

This does not exclude the possibility, however, that some type-I collagen may be formed in osteoarthrosis initiated by a different stimulus, particularly in more advanced phases of the disease. It is noteworthy that type-I collagen is the normal collagen component of articular cartilage in avian species (Eyre *et al.*, 1978).

Degree of glycosylation

Some of the differences in glycosylation of newly synthesized collagen between experimental and control knees probably reflect differences at the post-translational level in the quality of the type-II collagen molecules. In addition, the normal tissue may be synthesizing a significant amount of another type of collagen, besides type II as the main product, that is even more highly glycosylated. Recent findings support this latter explanation. Burgeson *et al.* (1979) have identified two new α-chains as minor components of human growth cartilage. By electrophoretic mobility and amino acid composition they appear related to αA and αB chains (Burgeson *et al.*, 1976) but analysis of CNBr-cleavage peptides indicates they are genetically distinct. Similar chains have been isolated from adult articular cartilage, in which they account for about 1% of the total collagen (Creasman & Eyre, 1980). These chains are much richer in hydroxylysine glycosides than is type-II collagen, and, moreover, are radioisotopically labelled when articular cartilage of adult rabbits is exposed to [³H]proline *in vivo* (D. R. Eyre, unpublished work). They may be a type of basement-membrane collagen located in the pericellular environment (Eyre, 1980).

Stimulated collagen deposition

In adult articular cartilage, collagen is turned over very slowly compared with proteoglycans (Maroudas *et al.*, 1976). The present study and previous radiochemical labelling experiments show that there is some synthesis and turnover, however (Repo & Mitchell, 1971; Eyre *et al.*, 1975; Maroudas *et al.*, 1976). The limited information available suggests that in osteoarthrosis both synthesis and breakdown of collagen are altered. In scarified articular cartilage of rabbits labelled with [³H]proline, Repo & Mitchell (1971) noted that the specific radioactivity

of hydroxyproline was higher than in control cartilage.

An increased concentration of procollagen was noted in the degenerative articular cartilage of dogs that developed spontaneous osteoarthrosis ('osteoarthritis') of the hip at an early age (Miller & Lust, 1979). No increase in the rate of synthesis of collagen was observed, but radioisotopic labelling was done with excised cartilage *in vitro*, where rates of synthesis probably do not reflect the situation *in vivo*.

The higher specific radioactivity of hydroxyproline in the pathological cartilage when proline was injected into the joint or given intravenously strongly suggests that the rate of synthesis and deposition of type-II collagen is increased 10–20-fold in early osteoarthrosis. This suggests that the potential of articular chondrocytes to repair damage to the collagen framework may be considerable.

The chondrocytes of normal articular cartilage display extremely low mitotic activity after skeletal maturity (Mankin, 1963), but a characteristic feature of naturally acquired human (Meachim & Collins, 1962) and canine (McDevitt *et al.*, 1977; Tirgari & Vaughan, 1975) osteoarthrosis, and of severe lesions in the experimental disease, is the appearance of groups of cells that have undergone mitosis (McDevitt *et al.*, 1977). It seems unlikely, however, that the increased deposition of collagen seen in the present work is solely related to an increased number of cells. The 10-fold increase at 2-weeks suggests an immediate effect on the metabolism of existing chondrocytes.

As all surfaces of articular cartilage in the operated joint, including the fibrocartilaginous menisci, expressed the response in unison, it appears that the cells were responding to some common stimulus. A diffusible substance, perhaps released from the synovial membrane during the post-operative synovitis, would seem to be a likely mediator of such an effect (Floman *et al.*, 1980).

Non-collagenous proteins

Proteoglycans and link protein are the principal constituents of 4 M-guanidinium chloride extracts of cartilage. Under associative conditions they separate at the bottom of the gradient on CsCl-density-gradient centrifugation (Hascall & Sajdera, 1969; Heinegård & Hascall, 1974), whereas proteins not bound to proteoglycans, including the small amount of soluble collagen, separate at the top (Bayliss & Ali, 1978; Inerot *et al.*, 1978). In the present experiments, this low-density fraction was the most highly labelled protein fraction of normal articular cartilage. The major component was of very high molecular weight, and on reduction with mercaptoethanol gave a single band on polyacrylamide-gel electrophoresis. This material accounted

for more than one-quarter of the total protein-bound radioactivity of normal cartilage labelled *in vivo* with [³H]proline and [³H]lysine together.

The results do not establish whether the highly labelled protein is a component of the cartilage matrix and synthesized by chondrocytes or is adsorbed on the articular surface from the synovial fluid. Preliminary analyses suggest the latter (D. R. Eyre, unpublished work). Whatever the source, the fact that this protein and non-collagenous proteins other than proteoglycans are heavily labelled suggests an important function in articular cartilage.

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