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Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage

I. Chemical composition

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SUMMARY Radiochemical and biochemical methods were used to characterize post-mortem and osteoarthrotic femoral head cartilage. Fixed charge density measurements were correlated with glycosaminoglycan content as estimated by uronic acid and hexosamine analyses. In post-mortem cartilage water content decreased from a maximum at the surface to a minimum in the deep zones. In the osteoarthrotic specimens water content was greatest in the middle zones. Glycosaminoglycan content increased with depth and in the osteoarthrotic specimens was reduced throughout the depth of the cartilage. With increasing degeneration there was an increase in water content and decrease in glycosaminoglycan content. The difference in the water content profile in osteoarthrotic cartilage was explained in terms of damage to the collagen network. In osteoarthrosis the latter is no longer capable of restraining the swelling pressure produced by the glycosaminoglycans and swelling is greatest in the midzones, where glycosaminoglycan content is highest.

Topographical variations in the total glycosaminoglycan content of human cartilage has been studied using the fixed charge density (FCD) method in post-mortem specimens of the hip (Maroudas et al., 1973) and knee (Ficat and Maroudas, 1975). FCD measurements have been correlated with biochemical analyses for cartilage of the human femoral condyle (Maroudas et al., 1969). Chondroitin sulphate showed small variations with depth but keratan sulphate content increased with depth from the surface. Chemical analyses have also been correlated with the distribution of histochemical staining (Stockwell and Scott, 1967). A similar distribution pattern has been described for bovine knee cartilage when the glycosaminoglycans were separated as their macromolecules (Lempert et al., 1974).

The aims of the present study were first to confirm that the FCD as measured by the tracer cation method (Maroudas and Thomas, 1970; Maroudas et al., 1973) agreed over a wide range of concentrations with the values calculated from chemical determinations of uronic acid and hexosamine, and second, to compare topographical variations in the chemical composition of cartilage from normal femoral heads (obtained at post mortem) with those of osteoarthrotic cartilage from patients undergoing total hip replacement. In the latter investigation care was taken to dissect well characterized full depth plugs of cartilage to avoid the use of pooled material. It was thus possible to obtain and study cartilage which ranged from only slightly surface fibrillated to surface fibrillated or eroded, and hence to determine in detail the chemical characteristics at the different stages of cartilage degeneration.

Materials

Human femoral heads were obtained either at post mortem or at operation for total hip replacement. The age range of the patients was 51 to 77 years for both the post-mortem material and the osteoarthrotic cases. Full depth chunks of cartilage approximately 1 cm in diameter were excised from the femoral heads. In the case of post-mortem material all the chunks had an intact surface, as assessed by Indian ink staining (Meachim and Stockwell, 1973). Samples were taken, when the condition of the specimen allowed, from all four regions of the femoral head, i.e. superior, inferior, posterior, and anterior.

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In the case of osteoarthrotic heads the superior surface was usually denuded of cartilage while the region under the fovea contained osteophytic tissue and was therefore avoided. Hence the specimens were usually obtained from the anterior and posterior aspects. These specimens were of variable quality, ranging from visually normal or slightly surface fibrillated to severely and deeply fibrillated.

In order to study the variation in chemical composition with depth, some of the full depth chunks (19 chunks from 7 post-mortem heads) were cut into 200 μm slices parallel to the articular surface. Among the osteoarthrotic specimens, only those which were either intact or mildly fibrillated could be adequately sliced (15 samples from 8 femoral heads). Where comparison was carried out between normal and osteoarthrotic full depth chunks, only those from the posterior and anterior aspects were used.

**Methods**

The full depth pieces of cartilage were allowed to equilibrate at 4°C in Ringer’s solution and were weighed. The following measurements were then carried out.

**FCD**

FCD is defined as the concentration of negatively charged fixed groups in the tissue (on wet or dry basis) and is usually expressed in mEq/g. In this investigation FCD measurements were carried out by means of the tracer cation method (Maroudas and Thomas, 1970). This method consists of equilibrating a tissue specimen in a dilute NaCl solution (for cartilage 0.015 mol/l NaCl is usually used), containing 22Na in tracer quantities. Under these conditions free electrolyte is virtually excluded from the cartilage because of the Donnan equilibrium (Maroudas, 1970), the only ions left inside the tissue are the cations (Na+) balancing the negatively charged fixed groups. Thus, by measuring the concentration of Na+ one can obtain a value for the total concentration of negatively charged groups.

The experimental procedure was as follows. The cartilage specimen (full depth chunk or slice, as the case may be) was allowed to pre-equilibrate in a suitable volume of a 0.015 mol/l NaCl solution (approximately 100 times the weight of the specimen). The time required for pre-equilibration depends on the thickness of the specimen: for 200 μm slices a few minutes will suffice, while for full depth chunks (around 2 mm in thickness) a 4-hour period is usually allowed. After pre-equilibration the specimen was transferred into a 0.015 mol/l NaCl solution, labelled this time with the radioactive isotope 22Na. The same period of time is required for equilibration as for pre-equilibration, but it was often convenient to leave the immersed specimen overnight. The equilibrations were usually carried out at 4°C to minimize autolysis. Except for overnight experiments, all solutions were normally kept stirred.

After equilibration the specimens were taken out of the radioactive solution and after being gently blotted to remove adherent liquid, were transferred for the determination of radioactivity to a γ-scintillation counter. 1 ml aliquots of the ‘hot’ solution were counted at the same time.

FCD was calculated by the formula:

\[
\text{FCD (mEq/g) = Radioactivity in the specimen (cpm) \times solution concentration (mol NaCl/l)} / \text{Wet (or dry) weight of specimen (g) \times radioactivity per g hot solution (cpm)}
\]

Since the solution concentration appears in the above formula, it should be checked in each experiment. This is done most simply by measuring the electrical conductivity of the ‘hot’ solution used and referring to a calibration curve of conductivity versus solution concentration at a given temperature. It is also important to ensure that the pH of the solution remains above 6.0, as below this value the carboxyl groups in cartilage start discharging and there is a consequent decrease in FCD (Freeman and Maroudas, in preparation).

Finally, it should be pointed out that for FCD below 0.05 mEq/g wet tissue, the presence of the chloride ion in the tissue is no longer negligible and will lead to an overestimate of FCD. The discrepancy will increase as FCD decreases. At FCD of 0.05 the error is about 15%. Most of the FCDs observed in practice lie above 0.05 mEq/g. To determine accurately an FCD below this value the tracer method is modified using a solution containing both 22Na and 35Cl (Freeman and Maroudas, in preparation). The use of solutions of molarity below 0.01 is not recommended, as problems arise relating to rates of Na+ exchange.

**DETERMINATION OF WATER CONTENT**

There are several difficulties inherent in the determination of the water content of tissue such as cartilage. First, if the cartilage on the joint surface is exposed to the air even for a few minutes some evaporation will take place unless the operations are conducted at 100% humidity, and this is not always practicable. Provided the tissue does not swell when excised and immersed in physiological saline, the best method is to dissect out the specimens, leave them to equilibrate in the solution, and weigh them subsequently. In
preliminary experiments we weighed full depth samples of normal cartilage immediately upon excision from a freshly opened joint and subsequently after equilibration for varying periods of time in Ringer’s solution. It was found that the difference in weight was negligible (<2%) provided the original cutting operation was conducted very rapidly or at 100% humidity. Furthermore, there was little difference (<3%) in the weight of thin (200 μm) slices, whether the latter were weighed directly after being cut from a full depth piece or after being individually equilibrated in Ringer’s solution after cutting.

In the case of fibrillated specimens, whether from post-mortem or postoperative material, the cartilage could no longer be considered as a nonswelling tissue. It was found that a full depth piece weighed more when it had been equilibrated in Ringer’s solution than immediately after excision, but this difference did not exceed 20%. However, once cut into slices these gained up to 100% of their wet weight after soaking in Ringer’s solution. Accordingly, when the moisture content of fibrillated cartilage was determined, it was essential to weigh the slices as cut, without exposing them to solution. It was also preferable to weigh chunks as soon as excised, although in this case the error introduced by immersion in Ringer’s solution before weighing was not large.

In the case of severely fibrillated cartilage, a further difficulty arises. In view of the high hydraulic permeability of such cartilage, slight finger pressure applied to the specimen during blotting could result in some fluid being expressed from the cartilage. We have used a different procedure to check that our wiping technique was satisfactory. In this procedure, the wet weight did not have to be measured.

The principle of the method is that large molecules, such as serum albumin, are practically excluded from cartilage, the partition coefficients, even in severely fibrillated specimens (of FCD around 0·05), being <0·1. If a dried piece of cartilage is immersed in Ringer’s solution containing radioactively-labelled iodinated serum albumin, a change will be observed in the radioactivity of the solution as the cartilage takes up water without absorbing any of the serum albumin. Provided the weight of the hydrated cartilage specimen is not less than about 20% of the weight of the solution, it is possible to calculate with accuracy the final water content of the cartilage. The radioactivity per unit weight of the solution was measured before the experiment and after the cartilage specimen had come to equilibrium. The results obtained by the above technique and by direct weighing of wet cartilage were identical even in the case of severely fibrillated specimens, provided the latter were gently blotted with a piece of tissue without squeezing, before being weighed. The simple weighing procedure was therefore used in the bulk of our experiments.

**CHEMICAL ANALYSIS**

A dry weight of each sample was determined by drying to constant weight at 67°C. Each sample was digested in papain (1 ml/20 mg dry weight) overnight at 67°C (Hjertquist and Lemperg, 1967). The volume was made up to 10 ml with distilled water and 1 ml aliquots were taken for chemical analysis.

Duplicate samples of the papain digests were analysed for uronic acid content by an automated version (Von Berlepsch, 1969) of the Bitter and Muir (1962) procedure using glucuronolactone as a standard. Before hexosamine analysis, 1 ml samples were hydrolysed in stoppered test tubes in 6 N HCl for 4 hours at 100°C. The hydrolysed samples were dried in vacuo desiccators at room temperature. Standards of N-acetyl-glucosamine were also hydrolysed and dried with each batch of samples. Hexosamine content was determined by the Elson and Morgan (1933) reaction. The collagen content was determined after hydrolysis in 6 N HCl for 4 hours at 115°C. Hydroxyproline was determined using the Stegemann method (Stegemann, 1958) as modified for automated analysis by Grant (1964).

Glucose and galactose are constituents of cartilage collagen and these sugars also give a positive reaction in the determination of uronic acid (Bitter and Muir, 1962; Von Berlepsch, 1969). In samples with a low glycosaminoglycan content but high collagen content, the colour reaction produced by collagen may be of considerable significance. Cartilage collagen was given to us by Dr. L. Rosenberg. This was digested in papain and dialysed against distilled water overnight. Collagen and uronic analyses were performed on the dialysate. The ‘uronic acid’ content of the dialysate had a linear relationship to the hydroxyproline content of this fraction and corresponded to 4·2 μg uronic acid/mg collagen. These results were confirmed using an Amicon filtration apparatus and a PM 10 filter. A sample of shark skin chondroitin sulphate digested and dialysed showed less than 1% of the total uronic acid content in the dialysate. Human articular cartilage from which most of the glycosaminoglycan had been extracted using serial extractions with hydrogen peroxide, EDTA, and trypsin (Steven and Thomas, 1973) also gave a figure of 4·2 μg uronic acid/mg collagen in the dialysate. This correction factor was calculated from the collagen content of each sample and subtracted from the total uronic acid content. This is a small fraction of the total (<5%) in normal cartilage, but it becomes important in severely degenerate samples.
with a low glycosaminoglycan but high collagen content.

**Calculations**

Assuming that the total negative charge for keratan sulphate is one per disaccharide (one sulphate group), and that for chondroitin sulphate it is two per disaccharide (one sulphate and one carboxyl), the total charge (in mEq/g) attributed to keratan sulphate is \( x - y \) where \( x \) is the hexosamine content (mmol/g) and \( y \) is the uronic content (mmol/g). The charge contributed by chondroitin sulphate would be \( 2y \), and thus the total charge would be \( 2y + (x - y) = x + y \). In order to convert the chemical results into weights of keratan and chondroitin sulphate, for chondroitin sulphate the uronic results were multiplied by a factor of 513 (the molecular weight of the disaccharide) and for keratan sulphate \( x - y \) was multiplied by a factor of 464. Collagen content was calculated from the hydroxyproline content using a factor of 7.6 (D. R. Eyre, personal communication).

**Results**

**Correlation of fixed negative charge density with chemical analysis**

Slices of cartilage from post-mortem and osteoarthrotic specimens at different depths from the articular surface were used to obtain a wide range of glycosaminoglycan concentrations. In Fig. 1 the mean FCD is plotted against the mean charge calculated from the chemical results. A good agreement was observed, not only in samples with a high FCD (normal slices from the middle and deep zones), but also in surface slices with a lower FCD (0.09–0.12 mEq/g) and osteoarthrotic samples with a FCD down to 0.05 mEq/g.
GLYCOSAMINOGLYCAN (GAG) CONTENT IN DIFFERENT REGIONS OF NORMAL FEMORAL HEAD

Fig. 2A and B show typical values of cartilage thickness and FCD at different sites of the normal femoral head. Fig. 2A gives values for a 32-year-old, while 2B corresponds to the other extreme of the age range examined, i.e. a specimen from a 90-year-old. The thickest cartilage is found in the superior-anterior and the thinnest in the inferior-posterior region. Provided the cartilage surface is intact, the variations in total GAG content are small; they do exist, however, and follow the same pattern as the thickness variation. Thus, the thickest cartilage has the highest GAG content, while the thinnest site corresponds to the lowest GAG value.

VARIATION IN CHEMICAL COMPOSITION WITH DEPTH

Water content

The water content in post-mortem cartilage was highest (74% of the wet weight) in the superficial zone, decreasing with depth to about 67% in the deepest zone (Fig. 3). The osteoarthrotic specimens showed, apart from an overall increase in hydration, a different pattern of variation with depth: thus the water content is low in the surface zone, rises to a maximum in the middle zone, and falls again in the deep zone.

GAG content

The variation in GAG content with depth is shown in Fig. 4 on a dry weight basis and in Fig. 5 on a wet weight basis. In intact post-mortem cartilage the chondroitin sulphate content shows little variation with depth on a dry weight basis while the keratan sulphate increases with distance from the articular surface. In the osteoarthrotic specimens (most of which had some surface fibrillation), as far as the chondroitin sulphate level is concerned, there is a decrease near the surface as compared with normal but little difference in the middle and deep zones. There is, however, a more pronounced decrease in the overall keratan sulphate content in osteoarthrotic specimens which is observable throughout the depth of the specimen. When the results are plotted on a wet basis there is a more significant increase in the GAG concentration with depth and the difference between the normal and the osteoarthrotic specimen is more pronounced.
Collagen content
The collagen content in normal samples decreases from the surface to the middle zone and then increases again in the deep zone. In osteoarthritic specimens there is a uniform decrease in the collagen content from the surface to the deep zone (Fig. 6). The results for osteoarthritic specimens lie clearly below those for normal cartilage. The same pattern of variation with depth is observed on a dry basis. However, on dry basis the results for osteoarthritic specimens are not significantly different from those for the normal cartilage (Fig. 4).

Fig. 6 Variation in collagen content expressed on a wet weight basis as a function of distance from the articular surface for cartilage from post-mortem and osteoarthrotic femoral heads.

Fig. 4 shows that when the GAG and collagen contents are added together, a fraction of dry weight remains which is not accounted for. This fraction is very small (about 5%) in the surface layer of normal cartilage and reaches a maximum of 20% in the middle zone.

Differences in composition of full depth cartilage chunks as a function of the degree of degeneration
FCD was used as a quantitative measure of the degree of degeneration as it has been shown that it correlates very well with the degree of fibrillation as shown by Indian ink staining (Ficat and Maroudas, 1975) and histology (Byers, Venn, and Maroudas, unpublished).

Fig. 7 clearly shows the increase in the water content with decrease in FCD. The normal samples have a mean water content of 71-5, increasing to over 80% in severely fibrillated specimens. In the case of normal specimens no correlation between the water content and FCD was observed.

Fig. 7 Water content in full depth cartilage pieces from post-mortem and osteoarthrotic femoral heads as a function of FCD.

Fig. 8 shows the levels of chondroitin sulphate and keratan sulphate in both normal and osteoarthritic specimens at different values of FCD. In normal specimens the mean value of chondroitin sulphate is 0.05 mol/g wet weight, while that of keratan sulphate is 0.04 mol/g. Osteoarthritic samples with high FCD values (between 0.13 and 0.16 mEq/g, corresponding usually to an intact surface) had values for chondroitin sulphate and keratan sulphate content which were not significantly different from those of post-mortem specimens.

Fig. 8 Variation in chondroitin and keratan sulphate contents in full depth cartilage pieces from post-mortem and osteoarthrotic femoral heads as a function of FCD.
Discussion

**FCD VERSUS CHEMICAL ANALYSIS**

In previous studies it was found that the concentration of negatively charged groups in cartilage as determined by the streaming potential method (Maroudas et al., 1969) and later by the tracer cation method (Maroudas and Thomas, 1970), agrees well with the values calculated from chemical analysis of the GAG contents. However, the tracer cation method had been tested against the analytically determined values on a relatively small number of specimens only and these had all come from post-mortem knee cartilage. In view of the great advantages that the tracer cation method presents—it is extremely rapid, nondestructive, and can be used for tissue of any shape and practically any weight (from 1 mg to 1 g)—we considered it essential to investigate in more detail and over a wide range of values how accurately its results correspond with the GAG content. As shown in Fig. 1, the means of the experimental points lie extremely close to the diagonal through the origin (i.e. to the theoretical line based on a one-to-one correspondence between the two sets of values); an excellent agreement thus exists between FCD as determined by the tracer cation method and that calculated from chemical analysis, both in normal and osteoarthrotic specimens.

We may thus conclude that the tracer cation method can be used with confidence for the determination of the total GAG content. Moreover, our results imply that the majority of GAGs in cartilage show normal charge densities, even in osteoarthrosis. Thus, supersulphation or undersulphation, if at all present, cannot affect more than a very small fraction of the GAG population.

The fact that all the negatively charged groups of the GAGs are available to the sodium ions means that they are not neutralized internally, e.g. by the positively charged groups on the collagen fibres. This is entirely in agreement with the finding that at physiological pH the collagen fibres in cartilage have no net positive charge (Freeman and Maroudas, 1975).

**CHEMICAL COMPOSITION OF FEMORAL HEAD CARTILAGE AS A FUNCTION OF DEPTH**

As seen in Fig. 5, the variation with depth in the proportions of the various constituents of normal femoral head cartilage is similar to that previously reported for the femoral condyle by various workers (Maroudas, et al., 1969). However, the femoral head cartilage has a greater total GAG content than the knee (Ficat and Maroudas, 1975) and the proportion of keratan sulphate appears to be higher.

Provided fibrillated sites are avoided, there is relatively little difference between the various areas of the same femoral head. The GAG content, however, does appear to be somewhat lower on the inferior aspect, where the cartilage thickness also tends to be less. This is consistent with our previous observation for the femoral head (Maroudas et al., 1973) and for the cartilage of the patella (Ficat and Maroudas, 1975) and is being studied in more detail (Venn and Maroudas, in preparation).

If one compares the mean chemical composition of slices of normal and osteoarthrotic cartilage, one can see that on a dry weight basis there is no significant difference in the collagen content at any level and little difference in the chondroitin sulphate except in the surface zone. The keratan sulphate content, on the other hand, shows a lowering in osteoarthrotic specimens throughout the entire cartilage thickness. If in some of the specimens the surface layer is missing, this would imply an even greater reduction in the keratan sulphate at a given depth from the surface. A more pronounced decrease in keratan sulphate as compared with chondroitin sulphate is also indicated when full-depth chunks of osteoarthrotic cartilage are compared with normal chunks (Fig. 8), and this appears to be true for all levels of FCD, i.e. for all grades of degeneration. It must be observed, however, in this context that there is a greater scatter in keratan sulphate than in chondroitin sulphate values, whether normal or osteoarthrotic specimens are examined.

Although it is more or less generally agreed in the literature that cartilage degeneration is accompanied by a decrease in the total GAG content (e.g. Mathews, 1953; Bollet et al., 1963; Bollet and Nance, 1966; Mankin and Lippiello, 1971; Maroudas et al., 1973), there is divergence in the reports as to which of the GAGs is actually lost first. Our results are consistent with those of Meachim and Stockwell (1973) who found that in fibrillated knee cartilage, chondroitin sulphate is reduced to a lesser extent than keratan sulphate. Ali and Bayliss (1974) also reported a lowered ratio of hexosamine to uronic acid in osteoarthrotic hips as compared with normal. Mankin found a reduction in keratan sulphate in cartilage from osteoarthrotic hips, but unlike us, he claims to observe an actual increase in the total chondroitin sulphate. McDevitt and Muir (1976) observed as one of the earliest changes in experimentally induced arthritis in the dog a decrease in the glucosamine/galactosamine ratio, i.e. a decrease in the keratan/chondroitin sulphate ratio. On the other hand, a number of authors (Bollet and Nance, 1966; Hjertquist and Lemperg, 1972) have reported the opposite, namely that in fibrillated cartilage...
there is actually a greater reduction in the chondroitin than keratan sulphate. The reasons for these divergences are not clear at the moment. Different workers have used different sampling techniques and different methods of analysis and in our view artefacts in methodology may be responsible for this lack of agreement. The most significant difference between normal and osteoarthritic specimens is in their water content, both as regards the absolute level and the profile of variation with depth.

It has been known for some time that fibrillated cartilage has a higher water content than normal (Lindahl, 1948; Bollet and Nance, 1966). This at first was puzzling in view of the apparent contradiction between the fact that it is the highly hydrophilic GAGs which are responsible for the affinity for water exhibited by connective tissue and the observation that in fibrillated GAG depleted cartilage the water content is actually increased. However (Maroudas et al. (1973) have suggested the following explanation. The hydration of cartilage is determined by a balance between the swelling pressure of the GAGs and the restraining elastic force due to the collagen fibre network. If the latter is damaged the decrease in the restraining elastic force is greater than the decrease in the swelling pressure resulting from a loss of some GAGs and the tissue gains water.

The different hydration profile can be explained along similar lines. In intact cartilage the swelling pressure gradient must be balanced at each level by a tension in the collagen network (Maroudas, 1976). Once the latter is broken the water will distribute itself in the tissue in proportion to the local swelling pressure. Since the surface of cartilage loses its GAGs earlier than the deeper levels, it will show little tendency to swell. However, the middle zone with its still relatively high GAG content will imbibe more water as soon as the restraining force of its collagen fibre network is diminished. In the deep zone, on the other hand, the collagen network is still often intact so that the swelling tendency is effectively counteracted there. Further discussion on the swelling of degenerate cartilage will be given in part II of this paper (Maroudas and Venn, 1977).

The higher water content of degenerate specimens (increasing with the degree of fibrillation) implies that the decrease observed in the GAG content on a dry weight basis will be intensified when expressed per wet weight of tissue. This is clearly shown in Fig. 5 where the curves for osteoarthrotic specimens, both expressing the total GAG content and the individual chondroitin and keratan sulphates, lie below those for normal cartilage throughout the depth of the tissue. It should be remembered that it is the GAG content on the latter basis (or, more strictly, on a water basis) which determines cartilage properties such as fluid flow, ionic equilibria, swelling pressures, etc. Thus, the load bearing behaviour of the tissue, which depends on the latter properties, will be considerably affected (Maroudas, 1975a, b).

The collagen content, though showing little change on a dry weight basis (this is not surprising since collagen is the major component of the solids), is lower on a wet weight basis in osteoarthrotic specimens. This will possibly lead to a decreased tensile stiffness of the osteoarthrotic cartilage, though actual damage of the collagen fibre network is probably of far more importance in this respect.

References


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