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Glycosaminoglycan Turn-Over in Articular Cartilage [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1975 **271**, 293-313
doi: 10.1098/rstb.1975.0054

Phil. Trans. R. Soc. Lond. B. **271**, 293–313 (1975) [293]
Printed in Great Britain

Glycosaminoglycan turn-over in articular cartilage

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Glycosaminoglycan turn-over has been studied both *in vivo* and *in vitro*, by using sodium [^{35}S]sulphate as a precursor. The *in vivo* experiments were performed on rabbits and dogs, taking special care to monitor the ^{35}S radioactivity in the serum throughout the experiment and to measure the radioactivity due to unincorporated inorganic [^{35}S]sulphate in cartilage at the end of each experiment, in addition to that due to incorporated sulphate.

The inorganic sulphate content of the serum was also determined as well as the distribution coefficient for the inorganic sulphate ion between cartilage and serum. From this information it was possible to calculate accurately the rate of sulphate uptake by cartilage *in vivo* and hence the turn-over rate. Experiments were then performed *in vitro* on cartilage from rabbits and dogs and the *in vivo* and *in vitro* results were compared. A very good agreement was obtained between the two sets of results. Studies were then carried out under exactly the same *in vitro* conditions on human articular cartilage and it was thus possible to obtain a turn-over rate for the latter which one could trust was close to the actual *in vivo* value. The mean half-lives thus obtained varied from 45 days for the young rabbit to 150 days for the adult dog and 800 days for the human femoral head. In human cartilage there were considerable variations in turn-over rate within a single joint as a function of depth below the surface, and between different joints. Thus, while the mean half-life for the human femoral head is 800 days, that for the femoral condyle is 300 days. Cartilage from osteoarthrosic femoral heads did not appear to differ much with respect to sulphate uptake from the normal specimens although the turn-over rates were somewhat higher.

Introduction

The turn-over of the matrix of connective tissue, particularly with respect to its proteoglycan component, has been studied now for some twenty five years, both *in vivo* and *in vitro*.

The *in vivo* studies have been carried out on various small animals, the usual procedure being to inject a radioactive precursor (most frequently [^{35}S]Na_2SO_4) into a group of animals, to kill the latter at various time intervals therafter and to obtain a time course of radioactive decay for the various tissues involved.

Bostrom (1952) was the first to obtain by this method a value for the half-life of ^{35}S in costal cartilage of adult rats, namely, 17 days. Schiller, Mathews, Cifonelli & Dorfman (1956) found that turn-over rates, using ^{14}C precursors, were similar to those obtained with ^{35}S and concluded therefrom that turn-over studies with ^{35}S measure the metabolism of the entire chondroitin sulphate molecule. Gross, Mathews & Dorfman (1960) further discovered that both the protein and the polysaccharide portions of the proteoglycan complex also metabolize at the same rate. The latter authors also observed that the disappearance of tracers from cartilage does not follow first order kinetics and pointed out that this suggests the presence of more than one metabolic pool of proteoglycans in cartilage.

Davidson & Small (1963) in a series of papers investigated the decay of radioactivity in different proteoglycan fractions of skin, nucleus pulposus and costal cartilage of rabbits after injection of ^{14}C-labelled glucose and ^{35}S-labelled sodium sulphate as precursors. They also

found that the proteoglycan pool in cartilage was far from being homogeneous and that there were considerable differences between both the rate of labelling and decay of the 'soluble' proteoglycan fractions, and of the 'insoluble' residue. Half-lives of about 10–20 days were indicated for the soluble fractions while the half-life of the insoluble residue was estimated to be of the order of 4 months.

More recent and sophisticated studies of different metabolic pools in cartilage, such as those due to Rokosova & Bentley (1973) and Lohmander, Antonopoulos & Friberg (1973), all confirm the existence of several pools. Attempts have been made by the above authors to distinguish between the various pools by separating the proteoglycans according to size, extractability and type of disaccharide unit involved. Lohmander *et al.* (1973) have tried to resolve the non-linear semilogarithmic decay curve into linear components and hence to calculate the half-lives of the different pools. They found in young guinea pig cartilage (costal, nasal and the nucleus) that there were fast pools with half-lives around 4–5 days and slow pools with half-lives of 40–120 days.

While it is clear from the above studies that there are many proteoglycan pools in cartilage, some of which are turning over rapidly, it is impossible to tell merely by following the course of radioactive decay what fraction of the total proteoglycans these pools constitute. In order to answer the latter question it is necessary to determine the actual overall rate of synthesis of sulphated glycosaminoglycans and it is this aspect which we have been examining in the present study.

Parallel with the *in vivo* work, a number of investigations have been carried out in the past *in vitro* on fresh cartilage and these have often been concerned with human material (see, for example, Collins & McElligott 1960; Collins & Meachim 1961; Meachim 1964; Bollet & Nance 1966; Mankin & Lippiello 1970). The purpose of these studies has usually been to compare normal and pathological tissue, and the results have been used in a strictly comparative manner, without any attempt being made at relating them to *in vivo* turn-over data.

The purpose of the present study was to determine the rates of sulphate incorporation *in vivo* in experimental animals and to compare the results with those obtained *in vitro*. In this manner the average turn-over rates of the proteoglycans could be calculated both for the cartilage from the experimental animal and the human and a rational basis could be provided for the *in vitro* work on the latter.

If one wishes to estimate the rate of synthesis of sulphated glycosaminoglycans from ^{35}S incorporation experiments *in vivo*, it is essential to know both the concentration of inorganic sulphate in cartilage and the radioactivity due to the latter during the incorporation period. Alternatively, if the cartilage is in equilibrium with blood serum, it is sufficient to monitor the radioactivity due to ^{35}S in the serum.

Accordingly, as an essential part of the present investigation, determinations were made of the inorganic sulphate content of the serum and the radioactivity due to inorganic sulphate in the serum at frequent time intervals during each experiment.

Materials and methods

For the *in vivo* experiments two sets of experimental animals were used:

(i) Young New Zealand albino rabbits, weighing between $2\frac{1}{2}$ and $3\frac{1}{2}$ kg; also, two adult albino rabbits, weighing 4 kg.

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(ii) Adult greyhound dogs, weighing around 25 kg.

Five young and two adult rabbits and five dogs were used in the *in vivo* experiments and the same number in those *in vitro*.

The cartilage from the femoral condyles, femoral heads and humero-ulnar joints was analysed.

Human articular cartilage came from three sources:

(i) Fresh post-morten specimens (these consisted of femoral heads, femoral condyles and patellae).

(ii) Normal femoral heads obtained at operation for femoral neck fractures.

(iii) Osteoarthrosic femoral heads obtained at operation for total hip replacement.

(*a*) In vivo *procedure*

The rabbits were given an intravenous injection containing 1 mCi of carrier free sodium [^{35}S]sulphate in saline solution per kilogram body mass.

Intravenous rather than intra-articular administration of tracer was chosen because in this way the concentration of inorganic sulphate in cartilage did not vary too much during an experiment, being in equilibrium with blood practically throughout. On the other hand, if ^{35}S were injected directly into the articulation for a very short initial period of time the cartilage would be in contact with a highly radioactive solution, but this would rapidly become less and less radioactive until a steady state value equal to about one thousandth of the initial concentration was reached. Under such conditions it would be impossible to estimate the mean radioactivity due to inorganic ^{35}S in cartilage during an experiment.

The rabbits were killed after periods ranging from 30 min to 6 h after the injection and blood samples were taken at $\frac{1}{2}$ h intervals throughout each experiment to measure the radioactivity. After death several joints were excised as rapidly as possible, the cartilage was carefully dissected, weighed and the inorganic sulphate desorbed in a known volume of saline. The desorbate was then 'counted', so that the distribution coefficient for inorganic sulphate could be determined, as previously described (Maroudas 1970; Maroudas & Evans 1974).

The total glycosaminoglycan content of the cartilage was subsequently determined by the fixed charge density method (Maroudas & Thomas 1970; Maroudas, Evans & Almeida 1973) and the cartilage was finally digested with papain and counted again, to obtain the radioactivity due to the sulphate, incorporated into the glycosaminoglycans. The details of the method are the same as those previously published for the human cartilage (Maroudas & Evans 1974).

The proportion of chondroitin to keratan sulphate was determined on a limited number of cartilage specimens by estimating the uronic acid and hexosamine contents by the method of Bitter & Muir (1962) and Antonopoulos, Gardell, Szirmai & de Tyssonsk (1964) respectively.

The procedure used with dogs was similar except that they were kept under nembutal anaesthesia throughout the experiment. The experiments lasted from 1 to 4 h.

The fresh blood samples were centrifuged at 3000 rev/min and the serum diluted 1:100 with distilled water and 'counted' to obtain the radioactivity due to ^{35}S. From each animal a serum sample was also dialysed to assess the percentage of non-dialysable sulphate and all ^{35}S counts were then corrected accordingly.

Inorganic sulphate content in the serum was determined by the method of Miller *et al.* (1961), suitable for small serum samples.

(*b*) In vitro *experiments*

(i) *Animal material*

The animals were killed, their joints excised and the cartilage was removed and kept moist at 4 °C until the start of the incubation. The time which elapsed between the sacrifice and the beginning of incubation varied between 1 and 6 h and was found to have no measurable effect on the subsequent incorporation rate.

The incubation was carried out at 37 °C in (*a*) the animal's own serum, and (*b*) in Eagles' medium, with added penicillin and radioactive tracer ^{35}S. The concentration of the sulphate ion in the medium was 0.8 mM (the same as in the serum) and the molar activity was a count of 1.2×10^8 min^{-1} μmol^{-1} of sulphate.

The times of incubation were varied from 2 to 16 h, but the majority of experiments were carried out for 5–6 h as it was shown that the rate of incorporation remained constant between approximately $\frac{1}{2}$ h and 10 h (see figure 3; also Maroudas & Evans 1974).

After incubation, the cartilage was treated in the same way as has been described above for *in vivo* experiments.

(ii) *Human material*

From each cadaver, specimens from the femoral head and the femoral condyle were examined, as well as in a few cases specimens from the patella. The post-operative material consisted of femoral heads only.

In all cases the cartilage was incubated within 2–6 h of death or operation.

Full-depth specimens, approximately 6 mm in diameter, were cored out carefully. The incubation was always carried out on full-depth specimens since fine slicing must necessarily result in some cells being damaged (Maroudas 1975*a*).

The specimens were carefully examined visually and characterized as intact, surface fibrillated or showing deep fibrillation. Even in the case of osteoarthrosic heads, it was often possible to find small areas of visually intact or only mildly surface fibrillated tissue. Adjacent samples were removed for histology. Care was taken not to include fibro-cartilage amongst the specimens studied.

The incubation was carried out as described for dog and rabbit cartilage except for an initial equilibration period, which will be mentioned later on.

After incubation the specimens were left to soak in 2–3 changes of Ringer's solution to remove inorganic [^{35}S]sulphate.

The concentration of fixed negatively charged groups, expressing the total glycosaminoglycan content, was then determined on each specimen by the tracer cation method (Maroudas & Thomas 1970; Maroudas *et al.* 1973). This method consisted briefly, in equilibrating the specimens in 0.015 M NaCl solution containing ^{22}Na as tracer and in comparing the radioactivity of the specimen with that of the solution. The full-depth samples were subsequently cut into 250 μm slices parallel to the articular surface, weighed and 'counted' individually. In this way the concentration of negatively charged fixed groups could be calculated for each slice separately.

The slices were finally washed free of ^{22}Na and digested with papain. The digests were then counted in a β scintillation counter, as previously described (Maroudas & Evans 1974); the original solutions in which incubation had taken place were suitably diluted and also counted at the same time.

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Methods of calculation

(*a*) *Distribution coefficient of the inorganic sulphate ion between cartilage and* (1) *blood serum in* in vivo *experiment*, (2) *incubation medium in the* in vitro *experiments*

The molar distribution coefficient, K, is defined as the ratio of the inorganic sulphate concentration in cartilage to that in solution and is calculated by means of the formula

$$K = \frac{\bar{C}}{C} = \frac{N_1 V_1}{N_0 M_1}, \tag{1}$$

where $\bar{C}$ is the concentration of inorganic sulphate in cartilage (mmol/g), C, the concentration of inorganic sulphate in the fluid in equilibrium (mmol/g), N_1, the detected radioactivity due to inorganic [^{35}S]sulphate per millilitre of desorbate, V_1, the volume of desorbate (ml), M_1, the net wet mass of cartilage (g), and N_0, the detected radioactivity due to ^{35}S in incubation medium or in serum at death.

(*b*) *Calculation of the rate of sulphate uptake*

The rate of sulphate uptake in the *in vivo* or *in vitro* experiments can be calculated by means of the following formula

$$Q = \frac{N_2 K C_0}{N_0 t}, \tag{2}$$

where N_2 is the detected radioactivity due to ^{35}S present as incorporated sulphate per gram of wet cartilage, N_0, the detected radioactivity due to ^{35}S present as inorganic sulphate per gram of cartilage (mean value for the duration of experiment), K, the molar distribution coefficient for inorganic sulphate, between cartilage and blood serum (or incubation medium), and C_0, the concentration of inorganic sulphate in serum (or incubation medium).

The product KC_0 gives in fact the concentration of inorganic sulphate in cartilage.

It is obvious that it is not possible to measure an average value of N_0 for a given animal, since N_0 can only be obtained at the end of an experiment. However, if experiments are carried out on a number of animals for different periods of time, care being taken to inject exactly the same amount of radioactivity per unit body mass and to keep all other experimental conditions identical, one can assess the variation of N_0 with time and hence estimate a mean value of N_0 over a given period, for one animal.

If, as in the case of young rabbits, the cartilage is very thin, the equilibrium between cartilage and serum is reached very rapidly. In such a case the following simpler formula can be employed to calculate sulphur uptake:

$$Q = \frac{N_2 C_0}{N_0' t}, \tag{3}$$

where N_0' is the detected radioactivity due to ^{35}S present as inorganic sulphate per millilitre of serum (mean value over the duration of experiment). N_0' can be calculated for each experiment by plotting the variation of ^{35}S count in the serum as a function of time (as in figure 1) and calculating the mean from the area under the curve.

In the *in vitro* experiments, cartilage specimens are exposed to solution from both sides and therefore reach equilibrium relatively quickly (table 1). Thus for our rabbit and dog cartilage, which never exceeded 1 mm in thickness, the equilibrium was attained in less than 15 min and

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equation (3) could be used, N_0 being constant in this case since the amount of ^{35}S used up during a run was negligible.

Some of the human specimens, particularly those from the patella, could reach more than 3 mm in thickness and since for the latter the equilibration time would be of the order of two hours, it was decided to adopt the following procedure. The cartilage was allowed to equilibrate at 4 °C in the radioactive solution (at this temperature cellular activity is very low) for a period of time calculated from the specimen thickness according to the data in table 1. After this initial equilibration period the sample was transferred to a bath at 37 °C for incorporation of ^{35}S into the cartilage matrix. This procedure made it possible again to use equation (3), without introducing any errors. (The idea of pre-equilibration was suggested to us by Mr Steven Moores of the Royal National Orthopaedic Hospital.)

TABLE 1.† EQUILIBRATION TIMES FOR CARTILAGE OF VARYING THICKNESS

cartilage thickness / cm	diffusion coefficient $10^6 \times \bar{D}$ / cm²/s	time required to achieve 90% of final equilibrium: one side exposed to solution	time required to achieve 90% of final equilibrium: both sides exposed to solution
0.5	—	18 h 24 min	4 h 36 min
0.3	3.0	6 h 40 min	1 h 40 min
0.25	3.0	4 h 36 min	1 h 9 min
0.20	3.0	2 h 56 min	44 min
0.15	3.0	1 h 40 min	25 min
0.10	3.0	44 min	11 min
0.05	3.0	11 min	3 min

† Taken from *Biochim. biophys. Acta* **338**, 272 (1974) with permission of the publishers.

From the rate of sulphate incorporation and the concentration of proteoglycan sulphate in the tissue it was possible to calculate the mean half-life of the proteoglycans‡ from the following formula:

$$\tfrac{1}{2}\text{ life (days)} = \frac{\text{proteoglycan sulphate content}}{2 \times 24 \times \text{hourly sulphate uptake}}. \qquad (4)$$

RESULTS

(*a*) *Inorganic sulphate and associated radioactivity in serum*

Figure 1 shows the variation in the radioactivity of rabbits' serum as a function of time. Our own data agree with those of Richmond (1959), as can be seen from the figure.

The decay of radioactivity, after the initial 10 min, is slow, reaching half the 10 min value after 6 h. It is thus easy to obtain a mean value for the counts over the duration of the experiment.

The radioactivity in whole blood was much lower than that in the serum, which agreed with the finding of Richmond & Hastings (1960) that blood cells excluded inorganic sulphate to a considerable extent.

Since it is the inorganic sulphate in the serum which is in equilibrium with that in various tissues such as cartilage, the radioactivity in the serum and not in whole blood was used in all our calculations.

‡ The assumption is made that sulphate turn-over is equal to the turn-over rate of the whole proteoglycan molecule.

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It was found that 95% of the radioactivity was in the form of dialysable sulphate, not associated with serum proteins. This again was in agreement with Richmond's results (Richmond 1959).

The inorganic sulphate content of rabbit's serum was found to be 0.7 mM.

The variation in the radioactivity of dogs' serum as a function of time is given in figure 2, for three typical experiments. The decay is semi-logarithmic after the first ½ h, which agrees with the results of other workers Richmond & Hastings (1960).

The same type of results were obtained for the radioactivity in whole blood as well as for the proportion of non-dialysable sulphate in the serum as in the case of rabbits' blood. The inorganic sulphate content of the dogs' serum was found to be 0.75 mM.

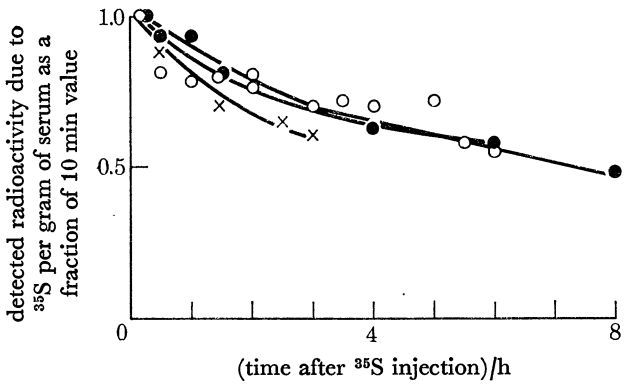

FIGURE 1. Decay of radioactivity from rabbits' serum after injection of ^{35}S. ○, ×, present data; ●, data due to Richmond (1959).

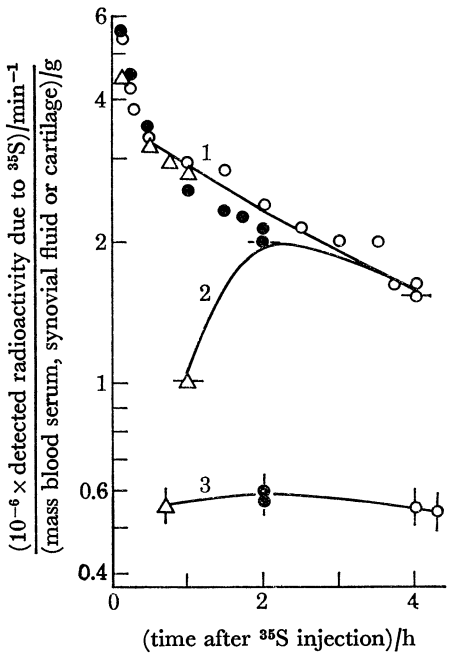

FIGURE 2. Change in the radioactivity due to inorganic [^{35}S] sulphate in blood serum, synovial fluid and cartilage after intravenous injection of ^{35}S into the dog. Curve 1, blood serum; curve 2, synovial fluid; curve 3 cartilage. △, 1 h experiment – blood serum; ⛛, 1 h experiment – synovial fluid; ⧋, 1 h experiment – cartilage; ●, 2 h experiment – blood serum; -●-, 2 h experiment – synovial fluid; ⧫, 2 h experiment – cartilage; ○, 4 h experiment – blood serum; -○-, 4 h experiment – synovial fluid; ◇, 4 h experiment – cartilage.

(b) Inorganic sulphate and associated radioactivity in cartilage and synovial fluid

Table 2 shows the mean ratios of counts due to inorganic [^{35}S]sulphate in cartilage to those in serum for the rabbits and the dogs sacrificed after different time intervals and also the mean distribution coefficients obtained *in vitro*.

TABLE 2. ATTAINMENT OF EQUILIBRIUM WITH RESPECT TO INORGANIC SULPHATE IN *IN VIVO* EXPERIMENTS

animal	duration of experiment/h	N_1/N_0	$K\dagger = \bar{C}/C_0$
rabbit	0.5	0.42	
	2	0.45	
	3	0.40	mean 0.44
	4	0.44	
	6	0.45	
dog	0.75	0.23	
	2	0.33	mean 0.36
	4	0.37	

† Obtained *in vitro*.

It is clear that in the young rabbit the two ratios are equal at all times and hence it can be inferred that equilibrium exists throughout the experiment between cartilage and serum. This is not surprising in view of the fact that in these young rabbits the cartilage was very thin, usually well below 0.5 mm and hence diffusion was rapid. Since the rabbits were immature, there could also have been diffusion from the bone (Maroudas, Bullough, Freeman & Swanson 1968).

On the other hand, in the dogs, after $\frac{3}{4}$ h, equilibrium was far from being achieved, the mean ratio N_1/N_0 being equal to 60 % of the value of the mean distribution coefficient. At 2 h and thereafter equilibrium appears to be complete. It should be stressed that the values given in table 2 are mean values for all three joints examined (elbow, femoral head, and femoral condyle). The rate of approach to equilibrium was found to vary from joint to joint, being slowest for the femoral condyle where the cartilage was the thickest (reaching sometimes 1 mm).

TABLE 3. INORGANIC SULPHATE CONTENT OF CARTILAGE

animal	sulphate ion concentration in serum water / μmol/g	mean molar distribution coefficient between cartilage and serum water	inorganic sulphate ion concentration in cartilage / μmol/g
young rabbit	0.7	0.44	0.31
adult dog	0.75	0.36	0.26
human adult:	0.6		
femoral head	—	0.30	0.18
femoral condyle	—	0.45	0.27

The rate of achievement of equilibrium appears to be somewhat slower than one would anticipate from table 1, but it is necessary to bear in mind that [^{35}S]sulphate has to reach the synovial cavity before diffusing into cartilage and this also takes time.

Figure 2 shows the radioactivity due to inorganic [^{35}S]sulphate at different times both in cartilage and in synovial fluid as well as in serum.

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It can be seen that, although equilibrium is not established at 45 min, the amount of radioactivity in cartilage does not change much over the period from $\frac{3}{4}$ to 4 h. Thus, because of relatively slow diffusion, cartilage does not mirror the steep initial drop in the radioactivity of the serum. It is therefore easy to use a mean value for N_0 in equation (2) for the purpose of calculating the sulphate uptake.

Typical values of the distribution coefficient, K, and the deduced values of the inorganic sulphate content of cartilage (KC_0) are given in table 3.

These values are based on both the *in vitro* and the *in vivo* results in the case of rabbit and dog cartilage, but for obvious reasons on the *in vitro* results only in the case of human cartilage.

The distribution coefficients for inorganic sulphate are seen to lie in the range 0.3–0.5, this being in accordance with Donnan equilibrium considerations and our previous results (Maroudas 1973). The variations are due to variations in fixed charge density.

The distribution coefficient and hence the sulphate concentration applies to cartilage as a whole. It is not known what the concentration of inorganic sulphate is inside the chondrocyte, but this knowledge is not required in the turn-over studies provided the equilibrium with respect to ^{35}S is reached rapidly between the chondrocyte and the matrix. That this is in fact the case is shown by the fact that plots of ^{35}S incorporation against time are linear, as will be illustrated in the next section.

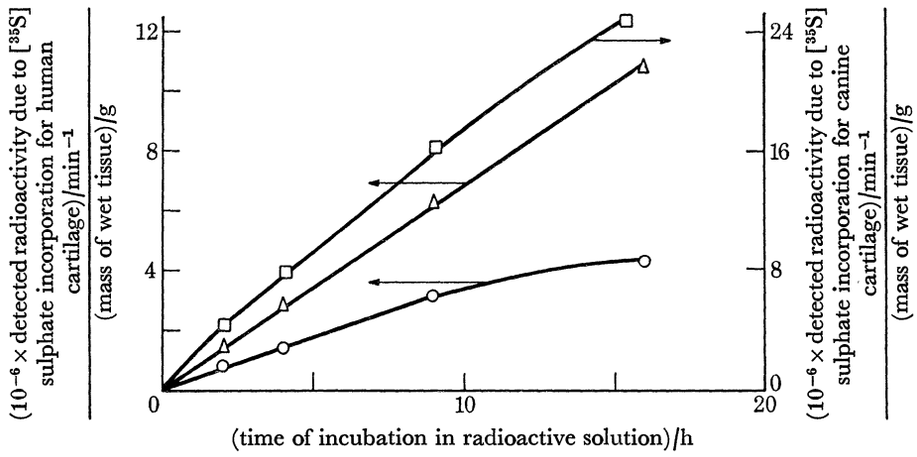

FIGURE 3. Variation of ^{35}S sulphate uptake into human and canine cartilage *in vitro*, as a function of incubation time. □, canine cartilage, femoral head; △, human femoral condyle; ○, human femoral head.

(*c*) *Sulphate incorporation into the cartilage matrix*

Figure 3 shows the variation in ^{35}S incorporation into the matrix of cartilage as a function of time obtained in *in vitro* studies on dog and human cartilage.

The cartilage had been pre-soaked for $\frac{1}{2}$ h in the radioactive solution at 4 °C before the start of incubation.

These curves show that the rate of ^{35}S incorporation remains constant throughout the first 10 h of experiment at least, and usually longer. This implies, among other things, that inorganic [^{35}S]sulphate inside the chondrocyte is in equilibrium with the inorganic [^{35}S]sulphate in the matrix throughout the experiment and that, accordingly, one is justified in using equations (2) and (3) to calculate the rate of sulphate uptake.

Figure 4 shows the variation in [^{35}S]sulphate uptake with time, *in vivo*, in the experiments with the young rabbits and the dogs.

There is a fair amount of scatter in the values for young rabbit cartilage, most probably because of age variations among the animals used. However, it is clear in the *in vivo* experiments too that the increase in sulphate uptake is linear with time.

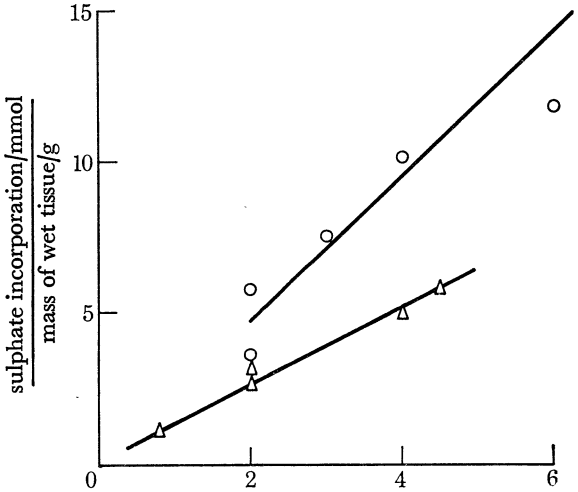

FIGURE 4. Variation in the sulphate incorporation into the articular cartilage of young rabbits and adult dogs, as a function of time after ^{35}S injection. ○, rabbit; ,△ dog.

(*d*) *Comparison between* in vivo *and* in vitro *sulphate uptake and turn-over rates*

Table 4 gives the mean rates of sulphate uptake for articular cartilage, calculated according to the above equations from *in vivo* as well as *in vitro* results.

No difference was observed between the results of *in vitro* incubation whether carried out in the animal's own fresh serum or in Eagle's medium.

Table 4 shows that in the case of the dog cartilage there is no significant difference between the sulphate uptake obtained *in vivo* or *in vitro*. In the case of rabbit cartilage, the uptake *in vitro* is 30 % lower than *in vivo*, most probably because the rabbit cartilage was very thin and difficult to excise without inflicting some damage on the cells.

The average half-lives, listed in table 4, vary from 45 days for the young rabbit to 800 days for adult human cartilage from the femoral head.

(*e*) *Sulphate turn-over in healthy human articular cartilage from different joints and at different depths from the surface*

Figures 5–7 show typical plots of fixed charge density, sulphate incorporation rate (represented by detected radioactivity per unit mass of tissue) and effective turn-over rate (represented by detected radioactivity per unit fixed charge density) respectively, against depth below the articular surface, for cartilage from the femoral head, femoral condyle and the patella.

Several points should be noted. First, as has been previously observed (Maroudas *et al.* 1973; Maroudas & Evans 1974; Ficat & Maroudas, in preparation) fixed charge density is considerably higher in the femoral head than in the knee, throughout the cartilage depth, while sulphate incorporation is lower. Secondly, for any given full-depth cartilage

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TABLE 4. SULPHATE INCORPORATION INTO ARTICULAR CARTILAGE

animal	number of animals tested	conditions of test	$10^5 \times$ rate of sulphate uptake/mmol h^{-1} / mass of tissue/g	proteoglycan sulphate/mmol / mass of wet cartilage/g	mean half-life of sulphated proteoglycans / days
young rabbit	5	*in vivo*	2.4 ± 0.05	0.055	47
(3-5 months)	5	*in vitro*	1.7 ± 0.03	0.055	67
old rabbit (2 years)	2	*in vivo*	1.3	0.060	100
adult dog	5	*in vivo*	1.3 ± 0.02	0.085	140
(greyhound)	5	*in vitro*	1.2 ± 0.015	0.085	150
human					
baby (5 weeks)	1	*in vitro*	1.7	0.10	130
adult femoral head	15	*in vitro*	0.2 ± 0.025	0.080	800
adult femoral condyle	15	*in vitro*	0.33 ± 0.04	0.050	300

specimen there is a great variation in the turn-over rate, according to the distance from the articular surface. Thus, in the femoral condyle cartilage (figure 7), the turn-over rate varies from a count of 20×10^6 min^{-1} mmol^{-1} of fixed charge (i.e. approximate half-life of 400 days) near the articular surface, to a maximum count of 40×10^6 min^{-1} mmol^{-1} fixed charge (half-life 200 days) and down to 30×10^6 min^{-1} mmol^{-1} fixed charge (half-life 300 days) in the deep zone. An even bigger variation is observed in the cartilage from the patella.

The difference between the sulphate turn-over in the middle zone of the femoral condyle cartilage and that of the middle zone of the femoral head is nearly fourfold.

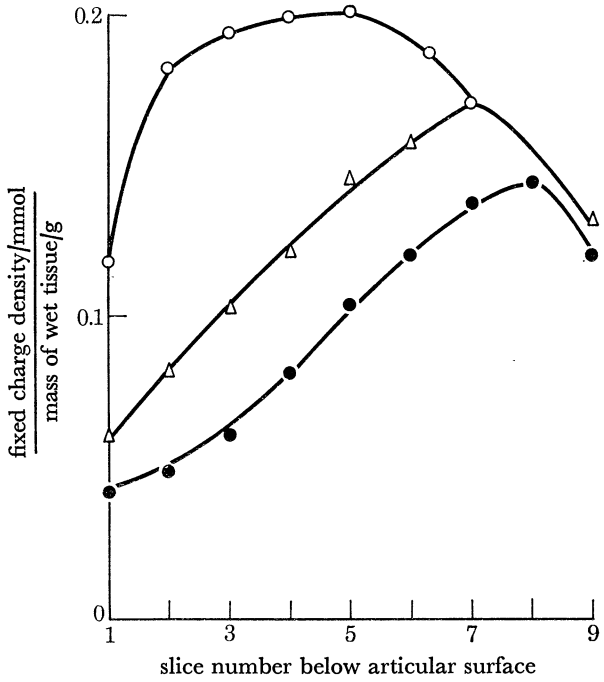

FIGURE 5. Variation in glycosaminoglycan content of normal adult human articular cartilage expressed as fixed charge density, plotted against distance below the articular surface. ○, femoral head cartilage; △, femoral condyle cartilage; ●, patella; age, 60 years.

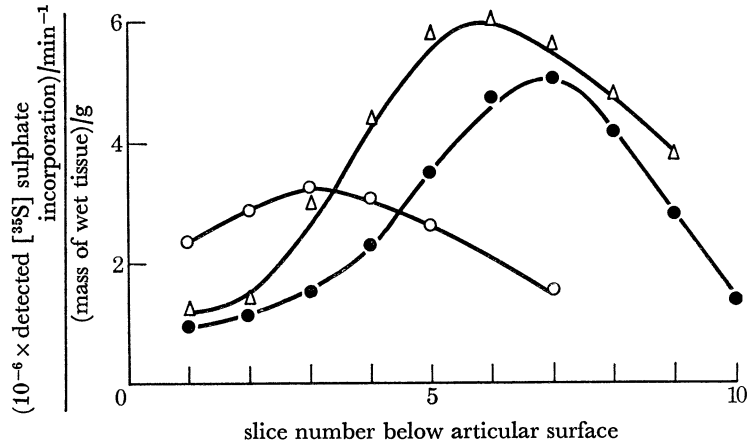

FIGURE 6. Sulphate incorporation for normal adult human articular cartilage during a five hour incubation period as a function of distance below the articular surface (key as in figure 5).

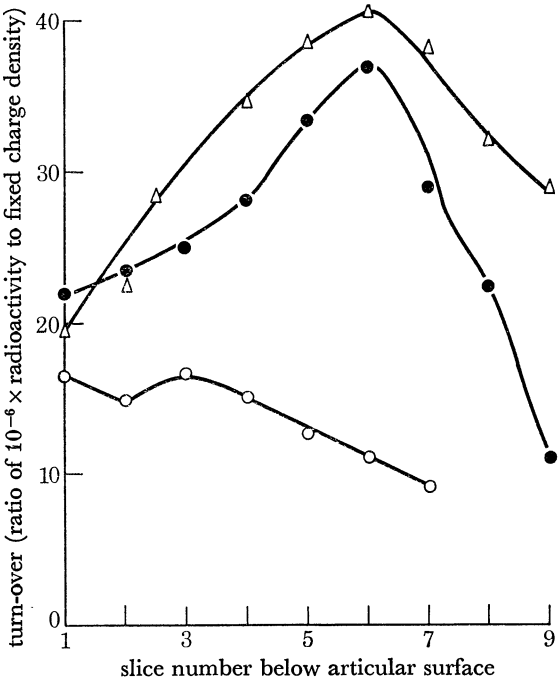

FIGURE 7. Variation in the turn-over rate as a function of distance below the articular surface (key as in figure 5).

The curves of radioactivity against fixed charge density represent true turn-over rates provided the proportion of keratan sulphate to chondroitin sulphate is low and does not vary much with depth. Typical curves of chondroitin and keratan sulphate contents against depth are shown for cartilage from the femoral head in figure 8 (Venn & Maroudas, in preparation). It can be seen that the keratan sulphate constitutes approximately one third of the chondroitin sulphate, which leads to a maximum error of only 15 % if one assumes the fixed charge density to be proportional to the concentration of sulphate groups throughout the cartilage depth. It is

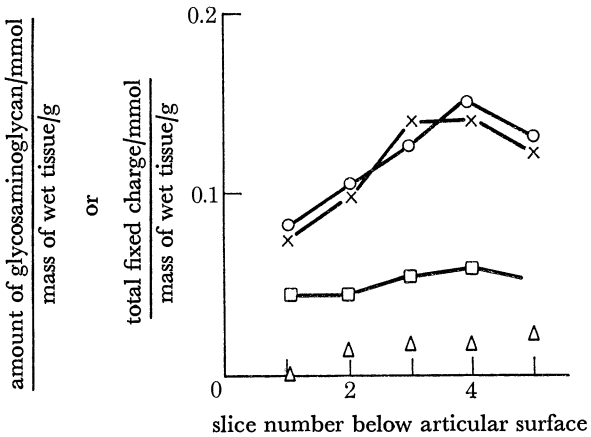

FIGURE 8. Variation in total fixed charge density as well as chondroitin and keratan sulphates as a function of distance below the articular surface, for normal human adult articular cartilage from the femoral head. □, chondroitin sulphate content; △, keratan sulphate content; ×, fixed charge density as measured by tracer cation method; ○, fixed charge density, as calculated from chondroitin and keratan sulphate contents.

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also assumed of course that the rate of turn-over of the sulphate group in keratan sulphate is not much higher than in chondroitin sulphate.

Figure 8 shows the agreement between fixed charge density, as obtained by the tracer cation method and as calculated from hexosamine and uronic acid analyses (also see Maroudas & Thomas 1970).

The shape of the curves of fixed charge density against depth (figure 5) differs somewhat for the femoral head and the knee. Although in both cases fixed charge density increases from the articular surface to the middle zone, dropping slightly in the deep zone, the curve for the femoral head rises much more steeply, reaching practically a plateau at 500 μm whereas the curves for the femoral condyle and the patella rise much more gradually and reach a maximum only at some 1400 μm from the surface. Similarly, the turn-over curves differ from the two joints: in the case of the femoral head there is usually a slight rise from the surface to 500 μm and the curve gently drops thereafter or there is a uniform slow decrease from the surface to the deep zone; in the case of the knee, the curves rise from the surface, reaching a maximum around 1000 μm and drop thereafter.

TABLE 5. COMPARISON BETWEEN INCORPORATION RATES IN NORMAL AND OSTEOARTHRITIC SPECIMENS (FEMORAL HEADS)

	age	fixed charge density mmol/g	10^{-3} × detected radioactivity $min^{-1}\ g^{-1}$	10^{-7} × radioactivity/f.c.d.
normal	84†	0.140	1700	1.20
	68‡	0.190	2000	1.05
	74‡	0.165	1700	1.02
	60‡	0.172	2150	1.25
	60‡	0.120	2500	2.10
	65†	0.140	1000	0.70
osteoarthritic	63	0.082	1700	2.10
	50	0.120	2500	2.10
	50	0.070	1400	2.00
	71	0.145	1700	1.17
	71	0.082	366	0.45
	66	0.067	1300	1.90
	60	0.142	1060	0.75
	60	0.067	570	0.85
	59	0.120	2800	2.30
	59	0.066	1014	1.50

† Femoral neck fractures, post-operative.
‡ Post mortem specimens.

(*f*) *Comparison between sulphate turn-over in normal cartilage and cartilage from osteoarthrosic femoral heads*

Table 5 shows the values of fixed charge density, sulphate incorporation and turn-over rates for five normal and six osteoarthrosic femoral heads.

Some of the specimens from the osteoarthrosic heads looked intact and showed a relatively high fixed charge density (around 0.14).

On the whole, the absolute incorporation rates per unit mass of wet tissue do not appear to differ much, whether they were obtained on specimens from normal or osteoarthrosic heads. It should be noted, however, that some very low incorporation rates were observed in some severely fibrillated specimens from osteoarthrosic heads.

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As far as the turn-over rates of the osteoarthrosic specimens are concerned, these lie on the average above those of the normal heads because of their reduced fixed charge density.

Figures 9–11 show typical curves of fixed charge density, incorporation rate and turn-over rate against distance from articular surface for specimens from two osteoarthrosic heads. Two of the specimens taken from two different heads (curves ● and □) showed considerable fibrillation while a third specimen (curve ×) taken adjacent to one of the others, exhibited only slight surface fibrillation. For the sake of comparison, curves for two specimens from a normal, *post-mortem* femoral head were also included. One of the normal specimens (curve ○) was completely intact while the other, which came from an area below the fovea, showed surface fibrillation (curve △).

It can be seen that curves ● and □ lie well below curve ○ for normal cartilage, with regard to fixed charge density and sulphate uptake, but not with respect to turn-over rate.

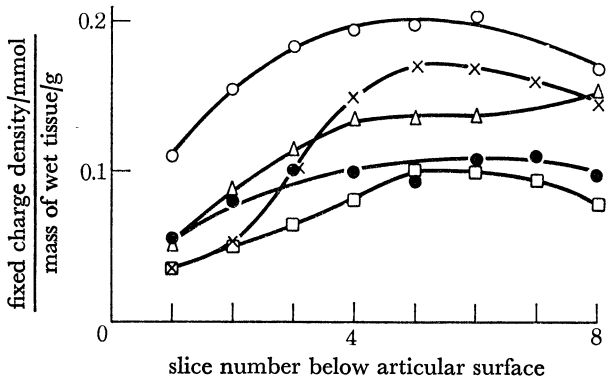

FIGURE 9. Comparison of fixed charge density versus depth curves for cartilage from normal and osteoarthrosic femoral heads. ○, post-mortem head, normal cartilage; △, post-mortem head, slightly fibrillated cartilage; ●, ostearthrosic head 1, fibrillated cartilage; ×, ostearthrosic head 1, slightly fibrillated cartilage; □, osteoarthrosic head 2, fibrillated cartilage.

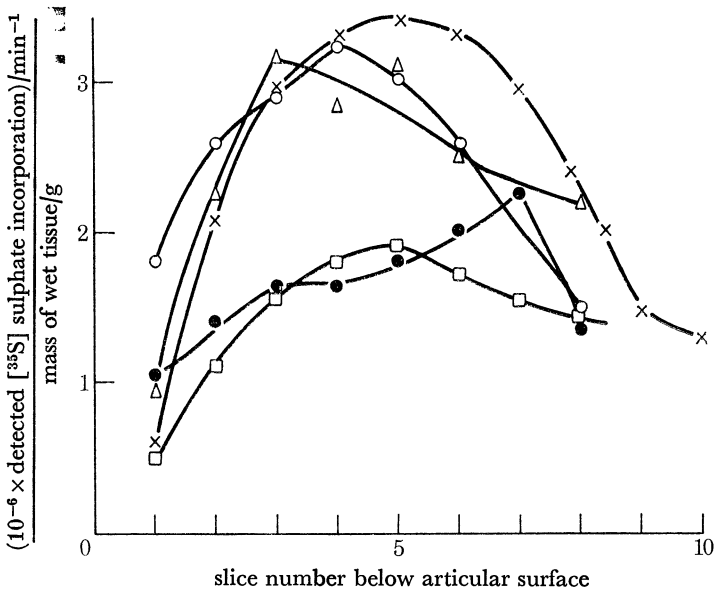

FIGURE 10. Comparison of sulphate uptake versus depth curves for cartilage from normal and osteoarthrosic femoral heads during 5 h incubation periods (key as in figure 9).

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However, curves △ and × (both of which exhibited only slight surface fibrillation but one of which came from a normal and one from an osteoarthritic head), lie close to one another as regards fixed charge density and sulphate uptake and practically coincide with regard to the turn-over rate.

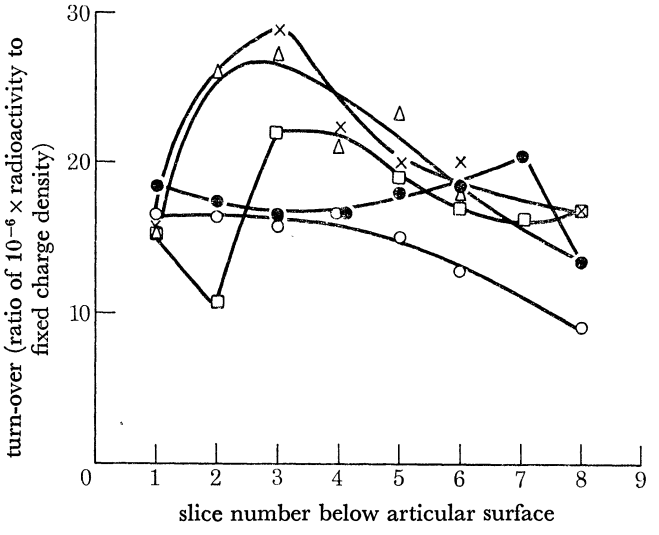

FIGURE 11. Comparison of sulphate turn-over against depth curves for cartilage from normal and osteoarthrosic femoral heads (key as in figure 9).

GENERAL DISCUSSION

(*a*) *Comparison between* in vivo *and* in vitro *sulphate incorporation*

The excellent agreement between the *in vivo* and *in vitro* data in the case of the dog is not really surprising. First, the dog cartilage was about 0.5 mm thick, so that intact full depth specimens could be cored out relatively easily, with a minimum injury to the cells.

Secondly, the maximum time from the removal of the joint to the start of the incubation period was 6 h and it has been demonstrated in the past that chondrocytes loose their sulphate fixing ability only very slowly, particularly when the tissue is kept at 4 °C (only 10 % of activity lost after 20 h, according to the data of McElligott & Collins 1960).

Thirdly, the rate of sulphate incorporation remained constant up to 10 h or more of incubation, which meant that within our normal period of testing the cells were not dying off.

There is thus no reason for the *in vivo* results to be different from those *in vitro*.

The 30 % discrepancy in the case of rabbit cartilage is most probably due to the unavoidable injury done to some cells when the very thin cartilage (0.2–0.3 mm) was being removed from the bone.

It is also worth noting that no difference was observed in the sulphate uptake whether the cartilage was incubated in Eagle's medium or serum. This means that if there are any sulphation factors, these must be within cartilage and cannot be easily removable.

(*b*) *Cartilage turn-over*

The mean turn-over rates of sulphated glycosaminoglycans from adult articular cartilage vary from 100 days for the adult rabbit to 800 days for the adult human femoral head. These

rates are much lower than has hitherto been thought; this is because there have been no rational quantitative measurements of total sulphate uptake in the past.

The low value for the mean turn-over of proteoglycans does not, of course, preclude the existence of fractions turning over at a faster or a slower rate than the mean, as indicated by the studies of the decay of radioactivity. For articular cartilage, for instance, the results of Mankin & Lippiello (1969) suggest the existence of a fraction with a half-life of 8 days. The present mean turn-over figures show clearly that such a fast fraction cannot constitute more than 8% of the total proteoglycans and probably less, as there must be a whole spectrum of proteoglycan molecules turning over at different rates.

It would be of great interest to know about the nature of the molecules in the different pools and it is tempting to speculate that the molecules in the fast pool should be smaller in size and/or less intimately associated with collagen. There is in fact some evidence in the literature – concerned with cartilages other than articular – suggesting that small proteoglycan molecules do have higher specific activities and hence higher turn-over rates than the larger species (Wasteson, Lindahl & Hallen 1972; Hardingham & Muir 1972; Rokosova & Bentley 1973).

Whatever the nature of the species involved in the different pools, the fact that the overall turn-over is slow implies that the balance between the anabolic and catabolic activity of the chondrocyte need not be controlled within too narrow a margin. The slow turn-over also makes sense physiologically if we remember that large molecules can only diffuse through cartilage with extreme difficulty (Maroudas 1970, 1975*b*).

The difference in the turn-over rate between the adult dog and the human could perhaps be directly related to the difference in the cellularity of the two tissues and inversely to the thickness (Stockwell 1971).

The turn-over in immature animals is much higher than in the adult and this again might be correlated with differences in cellularity (Stockwell & Meachim 1973). The finding of a higher turn-over rate in immature cartilage is consistent with the results of other workers (e.g. Mankin 1974).

(*c*) *Variations in sulphate uptake and turn-over in human cartilage*

(i) *Difference between the hip and the knee*

It has been found previously (Maroudas & Evans 1974) and confirmed in the present study that both the sulphate uptake and the turn-over rate are much higher in the femoral condyle than in the femoral head cartilage although the glycosaminoglycan content is lower. Since the thickness of cartilage is similar in the two joints, according to the work of Stockwell (1971), the cell density should also be similar. It must thus be the synthesis of glycosaminoglycans per cell which is higher in the cartilage having the lower glycosaminoglycan content.

The following feed-back mechanism is suggested, which might be one of the factors controlling the synthesis of sulphated proteoglycans.

It was found (Maroudas & Evans 1974) that sulphate incorporation increases with increasing sulphate concentration in the medium, over the physiological range of sulphate concentrations. But for a fixed concentration of inorganic sulphate in synovial fluid, the amount of inorganic sulphate actually present in cartilage varies inversely with its glycosaminoglycan content, in accordance with the Donnan equilibrium (Maroudas 1973). Therefore, the lower the glycosaminoglycan content in a specimen of cartilage, the higher will be its inorganic sulphate content.

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Because of the concentration dependence, one would thus expect the cartilage with the higher inorganic sulphate content to have a faster incorporation rate if all other relevant parameters are the same in both cases. Thus, the rate of glycosaminoglycan synthesis should be an inverse function of the total glycosaminoglycan content in the matrix of cartilage. Although we have only investigated the effect of sulphate concentration on sulphate uptake, other metabolically significant anions, such as phosphates, could act in a similar way as they too would be more or less excluded from the cartilage matrix, depending on the glycosaminoglycan concentration.

It has not yet been checked whether protein synthesis is also dependent on sulphate concentration or whether the latter only affects sulphate uptake. Such an investigation is being started.

(ii) *Zonal differences in uptake and turn-over*

It is clear from figure 6 that the rate of sulphate incorporation varies considerably, within a single joint, with distance from the articular surface. These local variations do not appear to be related either to cell density or to the glycosaminoglycan content in the same way as the gross joint to joint variations, since, at the surface, cell density is at its highest and the glycosaminoglycan content at its lowest, yet the rate of sulphate uptake is very much lower than in the middle zone.

The actual turn-over (represented by the ratio of sulphate uptake to fixed charge density) while varying somewhat less than the sulphate uptake, still gives, particularly in the case of the knee, a twofold variation within a millimetre of cartilage thickness. It is not known at present whether these local variations are due to the presence of different molecular species of proteoglycans in the different zones or to other factors, such as possibly different concentrations of lysozomal enzymes.

(*d*) *Comparison between normal and osteoarthrosic cartilage*

The results of this study, in which the number of specimens considered so far is rather small, show that where cartilage is considerably fibrillated, the sulphate uptake is often lower than in normal cartilage and even the turn-over rate is sometimes lower. Where cartilage is only mildly fibrillated, the sulphate uptake appears to be the same as in normal cartilage although the proteoglycan content is reduced. This results in a higher turn-over rate.

An important observation seems to be that there is no difference with regard to sulphur incorporation between cartilage specimens from a normal joint or an osteoarthrosic one, provided they exhibit similar degrees of fibrillation.

Our present findings are in agreement with our previous conclusions obtained purely on normal femoral heads (Maroudas & Evans 1974). They are also in agreement with the observations of Bollet & Nance (1966) who studied normal and fibrillated areas of the patella as well as with those of Lust, Pronsky & Sherman (1972) on normal and degenerative canine cartilage from the hip. There are some discrepancies, however, at the moment between our conclusions and those of Mankin & Lippiello (1970) who reported both higher sulphate uptakes and higher turn-over rates for osteoarthrosic cartilage. The reason for this difference is not clear at the moment. It is possible that we have not looked at a large enough number of o.a. specimens to date.

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Conclusions

By developing a procedure for the quantitative estimation of sulphate uptake in animals *in vivo*, it has been possible to compare the *in vivo* with the *in vitro* rates of sulphate incorporation and to show that there is a very good correspondence between the two.

It has thus been possible, for the first time, to obtain values for the mean glycosaminoglycan turn-over in man. The half-life for adult human femoral condyle cartilage is in the region of 300 days while that of the femoral head around 800 days, compared with a half-life of 150 days in the adult dog and 100 days in the adult rabbit. Cartilage from young animals exhibits a much higher turn-over of glycosaminoglycans than adult tissue.

A study of sulphate uptake and turn-over against distance from the articular surface has shown considerable local differences.

Cartilage from osteoarthrosic heads appears to have approximately the same sulphate uptake as the normal cartilage, but a higher turn-over rate.

This work was supported by a grant from the Medical Research Council.

The collaboration in different parts of this work of the following colleagues is gratefully acknowledged. Dr P. Byers, Dr C. Ficat, Mr S. Holm, Mr W. Souter and Miss G. Webber.

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Discussion

D. H. Leaback (*Institute of Orthopaedics, Stanmore, Middlesex*). Have you any information as to the nature of the glycosaminoglycans involved in your turn-over studies? I ask because I suspect that a considerable amount of your work has been carried out with relatively old cartilage and it is already known that such tissue has a fairly high proportion of keratan sulphate which turns over very slowly or not at all.

A. Maroudas. We are at present investigating the distribution of [^{35}S]sulphate in chondroitin and keratan sulphate by using cetyltrimethylammonium bromide precipitation and chondroitinase digestion. Our preliminary results indicate indeed that the turn-over of keratan sulphate is very much slower than that of chondroitin sulphate. However, since the fraction of keratan sulphate in human femoral head cartilage in the age range which we have been investigating is quite low – about 20 % of the total glycosaminoglycans (Venn & Maroudas, in preparation) – this affects very little our values of turn-over rates, which thus apply basically to chondroitin sulphate.

Since the relative proportion of keratan sulphate is highest in the deep layer of cartilage, the turn-over of chondroitin sulphate in that layer is in reality somewhat higher than that calculated on the basis of total glycosaminoglycans. The correction applicable to the superficial and middle zones is negligible.

R. A. Stockwell. How important is cell density in determining the rate of metabolic turn-over?

A. Maroudas. Cell density must certainly be an important parameter in determining the rate of metabolic turn-over of cartilage and the differences between the overall turn-over rates for rabbit, dog and human cartilage could well be accounted for by differences in cell density.

However, the differences between the sulphate uptake of the different joints of the same

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species do not appear to be explicable in terms of cell density. Thus in the human, the sulphate incorporation in the cartilage from the femoral condyle is usually significantly higher than that from the femoral head, yet the cartilage thickness in the two joints is similar, so the cell density should be similar.

Again, one cannot explain the differences between the turn-over rates of the different zones of articular cartilage in terms of cell density because the superficial zone, which, according to your work has the highest cell density, actually shows a low rate of sulphate incorporation compared with the middle zone.

Thus, while cell density must be an important factor in the metabolism of cartilage, there appear to be other parameters which in some cases override the effect of cell density alone.

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