Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage

II. Swelling

A. MAROUDAS AND M. VENN

From the Biomechanics Unit, Mechanical Engineering Department, Imperial College, London

SUMMARY Studies of the equilibrium, partition, and diffusion of tritiated water were carried out on normal and osteoarthrotic cartilage as well as on cartilage from which proteoglycans had been extracted chemically. All the water was found to be freely exchangeable in both normal and degenerate specimens. The diffusivity of the water was equal to about 40% of the value in free solution, with an activation energy equal to that in free solution. It was concluded that the swelling of degenerate tissue is not due to any changes in the state of the water, but to a failure of the damaged collagen network to oppose the swelling pressure of the proteoglycans. Swelling similar to that observed in fibrillated cartilage was also found in initially normal specimens treated with collagenase.

It has long been known that one of the symptoms of cartilage degeneration is an increased water content (see Bollet and Nance, 1966; Hjertquist and Lemperg, 1972; Maroudas *et al.*, 1973). This may seem surprising, for cartilage degeneration is usually accompanied by a loss of glycosaminoglycans (GAGs) which are themselves highly hydrophilic. One explanation for this apparent paradox is that a failure in the elastic restraint of the collagen network enables fibrillated tissue to swell to a higher degree of hydration in spite of the relatively low osmotic pressure of its decreased GAG content (Maroudas *et al.*, 1973; Maroudas, 1976). On the other hand, Mankin and Thrasher (1975) have suggested that when the GAG content is decreased some water binding sites on the collagen molecules are exposed and that consequently there is an increased content of 'bound', as distinct from 'free' water in fibrillated tissue.

The work described in this paper was undertaken in order to examine more closely these two suggested mechanisms by studying (a) the question of free and bound water in normal and fibrillated tissue, (b) the swelling behaviour of normal and fibrillated cartilage. The question of free versus bound water in cartilage

was studied by examining the equilibrium partition and diffusion of tritiated water.

Materials

Femoral heads obtained at post mortem as well as those removed for total hip replacement were used as the main sources of cartilage. Some samples from post-mortem patellae were also examined. Some of the cartilage specimens were treated with trypsin and hydrogen peroxide by the method of Steven and Thomas (1973) in order to remove the proteoglycans before experimentation. The level of residual proteoglycans in the latter specimens was extremely low (fixed charge density being no higher than 0.02–0.03 mEq/g wet tissue).

Methods

DISTRIBUTION AND DIFFUSION OF TRITIATED WATER

Principle of the method

If some of the water in a tissue is present in a 'bound' or 'structured' form, this will result in its being less mobile than the rest. The time taken for 'bound' water molecules to exchange position with the free water molecules in the immediate neighbourhood would be necessarily longer than if all the molecules were free. Thus, if one is examining the

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Correspondence to Dr. A. Maroudas, Bone and Joint Research Unit, Arthritis and Rheumatism Council Building, London Hospital Medical College, 25–29 Ashfield Street, London E1 2AD

overall rate of exchange in a tissue between two isotopes of water (e.g. ^3HHO and H_2O), water binding will reduce the effective rate of this process. Furthermore, if the slower exchange is limited to one fraction of the molecules only (i.e. if only part of the water is bound), it will not be possible to describe the overall exchange process mathematically in terms of a single diffusion coefficient. Accordingly, for the purpose of testing whether a portion of the water in either normal or fibrillated cartilage was present in a bound form, experiments were designed to study the rate of exchange of H_2O and ^3HHO between pieces of cartilage and Ringer's solution.

Experimental procedure

The diffusion coefficients were obtained from efflux curves determined in the following manner. Full depth cartilage specimens were left to equilibrate in Ringer's solution containing a trace of tritiated water. It was found in preliminary experiments that for a 2 mm thick piece of cartilage complete equilibrium was achieved in less than 2 hours at 37°C ; however, it was often convenient to leave the cartilage overnight in the radioactive solution at 4°C .

After equilibration the cartilage specimen was taken out of the radioactive solution and placed in 4 ml Ringer's solution. After a suitable time interval (the first interval being about 1 minute), the specimen was very rapidly* transferred to a second 4 ml aliquot of Ringer's solution. This procedure was repeated until there was no radioactivity left in the desorbate. Throughout the desorption process the solution in contact with the cartilage was vigorously stirred.

The radioactivity in each desorbate sample was found by 'counting' 2 ml aliquots, using a Triton based scintillation fluid, in a Nuclear Enterprises β counter. The radioactivity present per ml of the original equilibrium solution was determined at the same time.

In order to check that no radioactivity was left in the specimens after desorption, the latter were cut into two, one-half being digested in papain solution and 'counted', the other half being used for dry weight determination. The thickness of the specimens was measured by means of a micrometer and the measurements were usually checked using a microscope fitted with a graticule. Drying of samples was carried out to constant weight in an oven at 67°C .

Methods of calculation

The total radioactivity (ΣN) desorbed from a specimen was determined by adding the 'counts' present in the successive desorbates. A plot was

made of the fraction desorbed ($N_t/\Sigma N$) up to time t , versus $\sqrt{t}$, in accordance with the method given by Crank (1975). An efflux curve was thus obtained for each cartilage specimen. The diffusion coefficient was calculated from the early part of the curve using the formula given by Crank and Park (1968):

$$D = \frac{L^2 \times 0.0492}{t_{\frac{1}{2}} \times 60}$$

where $t_{\frac{1}{2}}$ is the time at which $N_t/\Sigma N = 0.5$ (t expressed in minutes); and L is the thickness of the specimen.

By comparing the values of the diffusion coefficient at two temperatures, it is possible to calculate the energy of activation for the given diffusion process, using the formula:

$$\frac{D_{T_1}}{D_{T_2}} = \exp \left(-\frac{A_D}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \right)$$

where D_{T_1} and D_{T_2} are the diffusion coefficients corresponding to the temperatures T_1 and T_2 respectively, A_D = activation energy, R = gas constant. For free diffusion processes the activation energy is usually in the range 3.0 – 6.0 kcal (12.5 – 25.08 kJ) (e.g. Crank and Park, 1968).

The advantage of the above procedure for the determination of the diffusion coefficients as compared with the steady state membrane method (Helfferich, 1964) previously used by one of us (Maroudas, 1968) is that the specimen does not need to be clamped in a diffusion chamber; this means that fibrillated specimens can also be tested. Furthermore, a comparison of the experimental efflux curve over its whole course with that calculated theoretically can yield valuable information on the diffusion process as a whole. However, for the sake of comparison, the steady state method was also used in a few cases.

The partition coefficients could be calculated from efflux results since the total amount of radioactivity taken up by each specimen at equilibrium was known, as was the radioactivity in the equilibrating solution. However, independent measurements of the partition coefficients were also carried out in the following manner. Full depth cartilage plugs were left in the radioactive solution just long enough for equilibrium to be achieved as calculated from the value of the diffusion coefficient, i.e. approximately 1 hour for a 2 mm thick chunk. The chunks were then cut on a freezing microtome into $300 \mu\text{m}$ slices, the latter were individually desorbed and the radioactivity present in the desorbates as well as in the equilibrating solution was determined. The cut slices were weighed after being dried at 67°C to constant weight.

* Transfer time being no more than 5 seconds.

The partition coefficients were calculated from the formula:

$$K_w = \frac{N_1}{N_0 \times W} \quad (3)$$

where K_w = partition coefficient for ^3HHO between cartilage and Ringer's solution expressed on a weight/weight basis, N_1 = radioactivity desorbed from cartilage slice, W = weight of cartilage slice, N_0 = radioactivity per g of equilibrating solution.

SWELLING STUDIES

The femoral heads, obtained either at post mortem or at operation for total hip replacement, were soaked in Ringer's solution. Full depth pieces of cartilage about 0.5–1.0 cm² were then excised and placed immediately in tared vials. The whole operation was carried out in a 100% humidity chamber to prevent loss of moisture during cutting.

In the case of fibrillated cartilage, specimens excised from the joint gain fluid over and above their original moisture content as soon as they are placed in contact with solution. This happens whether cartilage is immersed in solution as a full depth chunk or in thin slices. However, within the full depth chunk swelling is limited by the restraining effect of the collagen network in the deep zone, which is often intact in spite of surface fibrillation. Furthermore, the rate of imbibition of water into middle zone cartilage is much slower in a full depth chunk than in a thin slice because of the much reduced area of contact with the solution and a longer flowpath. Hence equilibrium is reached more slowly than in the individual slices (Fig. 5). The experiments carried out on slices are therefore more satisfactory than those done on full depth chunks for studying the swelling behaviour of the cartilage from the individual zones.

Accordingly, the full depth specimens were weighed after excision and were immediately sliced into 300 µm sections on a freezing microtome. The slices were weighed individually, soaked in 0.15 M NaCl solution, and reweighed. Constant weight was reached within 1–2 hours.

After reaching equilibrium with respect to 0.15 M NaCl, the slices were immersed in 0.015 M NaCl.

The equilibration procedure was repeated. The slices were finally dried at 67°C to constant weight.

COLLAGENASE TREATMENT

In order to test whether increased hydration would result if collagen were attacked enzymatically, a preliminary experiment was carried out *in vitro* in which cartilage slices were incubated in a buffer containing bacterial collagenase. The latter attacks the collagen molecule at both ends, giving rise to several fragments of different lengths (Stark and Kühn, 1968). It is specific for the Y-Gly bond in the sequence X-P-Y-Gly-P-Z (where P is proline or hydroxyproline and X, Y, and Z may be one of many other amino acids) (Grassmann and Nordwig, 1960; Bornstein, 1967). In native collagen the Y-Gly bone is cleaved, producing peptides with N-terminal glycine. The treatment of cartilage slices with bacterial collagenase would thus be expected to lead to extensive damage to the collagen network and solubilization of collagen fragments.

The experimental procedure was as follows. Full depth chunks of femoral head cartilage were cut into 250 µm slices parallel to the surface, weighed, and the total GAG content for each slice was determined by the fixed charge density method (Maroudas and Thomas, 1970; Venn and Maroudas, 1977). Slices from one chunk were incubated in 1 ml of 0.90 mg/ml collagenase (collagenase form II ex *Clostridium histolyticum*, Koch Light) in Tris 1% NaCl buffer pH 7.8 for 18 hours at 37°C. Slices from an adjacent chunk were incubated in buffer alone. After incubation the slices were reweighed and the fixed charge density was redetermined. They were desorbed and dried to constant weight at 67°C. The results were compared to the results for an adjacent chunk which had not been incubated.

Results

PARTITION COEFFICIENTS

The values of the partition coefficients of ^3HHO are given in the Table for 6 normal and 6 osteoarthrotic full depth pieces, and also for 2 pieces from which the proteoglycans had been extracted. In all cases there was excellent agreement between the ^3HHO

Table Diffusion and partition coefficients of tritiated water in cartilage (mean $\pm$ SD)

Type of specimen	No. of specimens	Diffusion coefficient (cm ² /s)		D_{37}/D_8	Partition coefficient	Fractional water content
		8°C	37°C			
Normal	6	6.55 $\pm$ 0.25	1.30 $\pm$ 0.035	1.98	0.7 $\pm$ 0.008	0.71 $\pm$ 0.003
Normal, without proteoglycans*	2	6.70	1.345	1.98	0.75	0.74
Fibrillated, from osteoarthrotic heads	6	7.35 $\pm$ 0.4	1.45 $\pm$ 0.065	1.98	0.76 $\pm$ 0.02	0.76 $\pm$ 0.02

* Proteoglycans having been removed by the trypsin EDTA–H₂O₂ treatment.

partition coefficient and the total water content as determined by drying the specimen. An equally good agreement is shown in Fig. 1 for the slices cut from chunks which had been equilibrated in ^3HHO for shorter periods of time (see procedure).

DIFFUSION COEFFICIENTS

The values of the diffusion coefficient for ^3HHO are given in the Table. The diffusion coefficient was slightly higher (about 10%) in the case of fibrillated specimens than in the normal, thus correlating with the higher water content. The ratio of the diffusion coefficient in cartilage to that in water lies in the range 0.45–0.50.*

Variation of diffusion coefficient with temperature

As the Table shows, the ratio of the diffusivities at 37°C and 8°C respectively is practically the same for all three sets of specimens. Hence the energy of activation, as calculated from equation (3), is also the same for the normal, the proteoglycan depleted, and the osteoarthrotic specimens. Its value is found to be 4.0 kcal (16.72 kJ), which is practically the same as in free solution (Mills, 1973).

EFFLUX CURVES

Typical experimental ^3HHO desorption curves are shown in Figs. 2–4. The initial portion of these

Fig. 1 Graph of the partition coefficient of tritiated water versus water content for 300 μm slices obtained from two surface fibrillated chunks of cartilage from osteoarthrotic femoral heads. $\times$ = specimen from patient aged 58 years; $\circ$ = specimen from patient aged 65 years.

* The value of the self-diffusion coefficient for ^3HHO in H_2O is 2.84×10^5 at 35°C (Mills, 1973).

Figs. 2–4 Efflux curves: graph of desorbed radioactivity versus square root of time for normal (Fig. 2), osteoarthrotic (Fig. 3), and trypsin treated (Fig. 4) specimens of cartilage. ($t_{1/2}$ corresponding to $N_t / \Sigma N = 0.5$ is obtained from each curve as shown in Fig. 1, and is used to calculate D , the diffusion coefficient, by means of equation 1.)

curves (up to $N_t/\Sigma N \approx 0.6$) is linear, in accordance with theory (Crank, 1975). The diffusion coefficient can thus be readily calculated from the value of t corresponding to $N_t/\Sigma N = 0.5$, as explained under Methods.

In order to find out whether the above diffusion coefficient, D , also describes the desorption process at values of $N_t/\Sigma N$ closer to unity, values of $N_t/\Sigma N$ were calculated for large values of t from the equation:

$$\frac{N_t}{\Sigma N} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n-1)^2 \pi^2} \exp \left\{ -D(n+\frac{1}{2})^2 \pi^2 t / L^2 \right\} \quad (4) \text{ (Crank, 1975).}$$

The experimental points lie very close to the calculated curves in all cases.

SWELLING OF FIBRILLATED CARTILAGE

A chunk of intact cartilage shows practically no change in weight when immersed in solution when

Fig. 5 Hydration of full depth specimens from osteoarthrotic heads as a function of time. $\times$ = fibrillated specimen from patient aged 70 years; $\square$ = superficially fibrillated specimen from same patient; $\circ$ = fibrillated specimen from patient aged 58 years.

compared with its initial weight as determined directly upon excision from the joint. On the other hand, chunks of cartilage whose surface is fibrillated show a gradual increase in weight when immersed in solution. This gain is slow, however, the equilibrium water content not being reached even after 24 hours (Fig. 5).

When the full depth chunks are cut into thin (250–400 μm) slices equilibrium is reached within 1–2 hours; the swelling is particularly significant in slices from the middle zone, as shown in Fig. 6. If the slices are then transferred into a hypotonic solution, e.g. 0.015 M NaCl, further imbibition of fluid takes place, again being most significant in slices from the middle zone.

The pattern of swelling as a function of depth parallels the hydration profile in fibrillated cartilage, as reported in part I of this study (Venn and Maroudas, 1977). Fig. 7 shows, for comparison, the variation of the GAG content on a wet and dry basis as a function of distance from the articular surface for a normal and a surface-fibrillated specimen.

COLLAGENASE TREATMENT

Slices incubated in collagenase showed an increase in water content throughout the depth of the cartilage despite a drop in the GAG content (Fig. 8b) and a decrease in dry weight. Slices incubated in buffer alone also showed some loss of GAGs but no increase in water content compared to the control group (Fig. 8a).

Fig. 6 Variation in the weight of water per dry weight of tissue versus distance from the articular surface for slices of osteoarthrotic cartilage. Δ = as cut; $\bullet$ = equilibrated in 0.15 M NaCl solution after cutting; $\circ$ = equilibrated in 0.015 M NaCl solution.

Fig. 7 Variation in glycosaminoglycan content with distance from articular surface in a normal and a degenerate specimen of cartilage, expressed both on dry and wet basis. The glycosaminoglycan content is expressed as the concentration of negatively charged fixed groups (FCD).

Discussion

The complete correspondence shown in the present study between the partition coefficients of tritiated water and the total water content (Table, Fig. 1) shows that all the water in cartilage, whether the latter be normal or degenerate, is freely exchangeable. This agrees with the previous results which showed that all water in cartilage is available to small solutes (Maroudas, 1970).

The diffusion coefficient for ^3HHO in cartilage is equal to 40–50% of its value in aqueous solution. This is the ratio found previously for other small solutes (Maroudas, 1970; Maroudas, 1975). This relative decrease in the value of the diffusion coefficient in cartilage as compared with free solution can be explained on a very simple mechanical model by two factors: (i) a smaller effective area being available to diffusion because of the presence of the solid matrix, and (ii) a more tortuous path for the diffusing molecules. The relation

$$\frac{\bar{D}}{D} = \{ (1 - V_p)/(1 + V_p) \} \quad (5)$$

(where $\bar{D}$ =diffusion coefficient within the polymer, D =diffusion coefficient in free solution, V_p =fractional volume of solids in the matrix) was derived by Mackie and Meares for homogeneous resins in which the network mesh width is large compared with the diameter of diffusing particles of small

Fig. 8 (a) Effect of collagenase on the hydration of cartilage slices. (b) Effect of collagenase treatment on the fixed charge density (FCD) of cartilage slices.

solutes (Crank and Park, 1968). In cartilage V_p varies slightly with the water content and the proportion of the various solid constituents, and lies in the range 15–20%.

The corresponding ratio $\bar{D}/D$, as calculated from equation 5 lies in the range 0.45–0.55. This is very close to the range of values of $\bar{D}/D$ actually observed. It can thus be said that the model of Mackie and Meares describes very well the diffusion of tritiated water and other small solutes in cartilage.

The small increase in the value of the diffusion coefficient which has been observed in fibrillated tissue as compared with normal is entirely consistent with its higher water content and lower GAG content, both of which factors lead to a lower value of V_p .

The fact that the desorption of ^3HHO can be described over its whole time course by a single diffusion coefficient in all three types of cartilage examined means that from the point of view of its mobility, all water in cartilage behaves as a one-compartment system whether the cartilage is normal, fibrillated, or artificially proteoglycan depleted.

The fact that the energy of activation is equal to that for diffusion in free solution also means that the water in cartilage is entirely free as far as the diffusional behaviour of its molecules is concerned and that this is so in both normal and degenerate cartilage. Our results thus in no way support the view expressed by Mankin and Thrasher (1975) that the excess water present in proteoglycan depleted specimens of cartilage is 'bound' water.

It should be noted that the rate of diffusion of any solute into or out of a specimen of tissue is very sensitive to the thickness of the specimen. Mankin and Thrasher, in their qualitative studies of the exchange of tritiated water, do not appear to have taken the specimen thickness into account: it is possible that the differences they observed in the rate of loss of ^3HHO between normal and degenerate cartilage were in reality due to differences in the shape of their specimens.

We suggest that the difference in the water content between the normal and the fibrillated specimens is due to the fact that in the latter the collagen network is impaired, its restraining force on the tissue is decreased, and hence fibrillated cartilage is able to achieve a higher degree of hydration in spite of the relatively low osmotic pressure of its decreased GAG content.

Our hypothesis is supported by the fact that while intact cartilage does not change its water content when the swelling pressure difference between it and the surrounding solution is altered, fibrillated tissue does swell when the osmotic stress is increased. Thus, as shown in Fig. 6, when a full depth chunk of

surface fibrillated cartilage is excised from the joint and cut into thinner slices parallel to the articular surface, and these are allowed to equilibrate in buffered saline (0.15 M NaCl), considerable swelling occurs in the slices from the middle zone, compared with the original water content. This is consistent with the increased osmotic stress in these slices when exposed directly to isotonic saline solution compared with the situation in the uncut joint. Thus, it must be noted that within the joint there is a gradual increase in the GAG content with distance from the articular surface and that it is the surface alone, with its low GAG concentration, which is in direct contact with synovial fluid. As a result there is a gentle grading of osmotic stresses within the tissue (Maroudas, 1976). When cartilage is excised from the joint, however, slices from each zone being exposed directly to saline solution, the swelling pressure gradient is considerably increased in regions where the GAG content is high; if the elastic restraining force of the collagen network is impaired in these regions the increased osmotic pressure gradient leads to water imbibition.

A further proof that once the collagen network is defective it is the osmotic pressure gradient which determines the hydration level is provided by the fact that swelling is further increased when the same cartilage slices are re-equilibrated in a hypotonic solution (see Fig. 6), since this leads to an increase in the ionic component of the pressure differential (e.g. Maroudas, 1975).

It is notable that when expressed on a dry weight basis, the concentration of GAGs in the surface fibrillated cartilage is lower than in the intact tissue only in the superficial zone. In the middle and the deep zones the GAG level is normal (see Fig. 7). This confirms our conclusion that increased hydration is not causally related to the previous loss of GAG since the swelling is most pronounced in slices from the middle zone where no such loss has occurred. On the contrary, in our view both the increased swelling and the loss of GAG from the surface could be the result of an impairment of the collagen meshwork.

Another explanation for the high water content of degenerate cartilage was put forward some years ago by Bollet and Nance (1966) who attributed it to a decrease in the mean molecular weight of the proteoglycans and hence an increase in the colloid osmotic pressure. This explanation is unlikely in view of the fact that the disaggregated proteoglycans are leached out of the tissue when the latter is immersed in isotonic salt solutions (e.g. McDevitt and Muir, 1976); yet in the present experiments no decrease in the swelling was observed as a function of time.

Although the results involving collagenase treatment must be treated as preliminary since only one experiment was performed, they show directly that damage to the collagen network results in an increased hydration of cartilage.

References

- Bollet, A. J., and Nance, J. L. (1966). Biochemical findings in normal and osteoarthritic articular cartilage. II. Chondroitin sulfate concentration and chain length, water and ash content. *Journal of Clinical Investigation*, **45**, 1170-1177.
- Bornstein, P. (1967). Comparative sequence studies of rat skin and tendon collagen. *Biochemistry*, **6**, 3082-3093.
- Crank, J. (1975). *The Mathematics of Diffusion*, 2nd ed., p. 50. Clarendon Press, Oxford.
- Crank, J., and Park, G. S. (1968). Methods of measurement. *Diffusion in Polymers*, p. 16. Ed. by J. Crank and G. S. Park. Academic Press, London and New York.
- Grassman, W., and Nordwig, A. (1960). Quantitativer Kolorimetrischer Test auf Kollagenase. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie*, **322**, 267-272.
- Helferich, F. (1962). *Ion Exchange*, pp. 314-317. McGraw-Hill, New York.
- Hjertquist, S. O., and Lemperg, R. C. (1972). Identification and concentration of the glycosaminoglycans of human articular cartilage in relation to age and osteoarthritis. *Calcified Tissue Research*, **10**, 223-237.
- McDevitt, C., and Muir, H. (1976). Biochemical changes in the cartilage of the knee in experimental and natural osteoarthritis in the dog. *Journal of Bone and Joint Surgery*, **58B**, 94-101.
- Mankin, H., and Thrasher, A. Z. (1975). Water content and binding in normal and osteoarthritic human articular cartilage. *Journal of Bone and Joint Surgery*, **57A**, 76-86.
- Maroudas, A. (1968). Physicochemical properties of cartilage in the light of ion-exchange theory. *Biophysical Journal*, **8**, 575-595.
- Maroudas, A. (1970). Distribution and diffusion of solutes in articular cartilage. *Biophysical Journal*, **10**, 365-379.
- Maroudas, A. (1975). Biophysical chemistry of cartilaginous tissues with special reference to solute and fluid transport. *Biorheology*, **12**, 233-248.
- Maroudas, A. (1976). Balance between swelling pressure and collagen tension in normal and degenerate cartilage. *Nature*, **260**, 808-809.
- Maroudas, A., and Thomas, H. (1970). A simple physicochemical micromethod for determining fixed anionic groups in connective tissue. *Biochimica et Biophysica Acta*, **215**, 214-216.
- Maroudas, A., Evans, H., and Almeida, L. (1973). Cartilage of the hip joint: topographical variation of glycosaminoglycan content in normal and fibrillated tissue. *Annals of the Rheumatic Diseases*, **32**, 1-9.
- Mills, R. (1973). Self-diffusion in normal and heavy water in the range 1-45°. *Journal of Physical Chemistry*, **77**, 685-689.
- Stark, M., and Kühn, K. (1968). The properties of molecular fragments obtained on treating calf skin collagen with collagenase from *Clostridium histolyticum*. *European Journal of Biochemistry*, **6**, 534-541.
- Steven, F., and Thomas, H. (1973). Preparation of insoluble collagen from human cartilage. *Biochemical Journal*, **135**, 245-247.
- Venn, M. F., and Maroudas, A. (1977). Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage. 1. Chemical composition. *Annals of the Rheumatic Diseases*, **36**, 121-129.