GELATIN AS AN EXPERIMENTAL MODEL FOR HYALURONATE

M. B. E. SWEET and L. SOLOMON, JOHANNESBURG, SOUTH AFRICA

From the Orthopaedic Research Laboratories, University of Witswatersrand, Johannesburg

The ground substance of articular cartilage exists in three phases: collagen, the protein-polysaccharide matrix, and the water and salts bound by the high negative charge density of the acid protein-polysaccharides.

The protein-polysaccharide molecule has been shown to consist of a central protein core to which are bound at regular intervals side chains of chondroitin sulphate (Mathews 1967) (Fig. 1). We have calculated the molecular weight of the substance to be \(7.8 \times 10^9\), using the Svedberg equation

\[
M_w = \frac{RT_s}{D(1-\nu p)}
\]

where \(R\) is the gas constant, \(T\) the absolute temperature, \(D\) the diffusion coefficient, \(\nu\) the partial specific volume and \(p\) the density of water at \(T\) degrees. This figure, however, should be treated with some reserve because of the non-ideality of the material. The substance can be extracted from cartilage without difficulty by stirring cartilage slices in 0.1 M acetate buffer at \(pH = 5\) with 0.85 per cent \(W/v\) sodium chloride at 37 degrees Centigrade for sixteen hours (Hoffman, Mashburn, Meyer and Bray 1967). Using high speed homogenisation in the cold, we have extracted even larger molecules from cartilage: these are probably aggregates of the smaller \(7.8 \times 10^9\) \(M_w\) “monomer”, held together by basic peptide bridges (Sweet 1968).

Considering the ease with which the monomer can be extracted by stirring slices of cartilage in the acetate buffer, it is remarkable that protein-polysaccharide is present in exceedingly low concentration in synovial fluid (Silpananta, Dunstone and Ogston 1967). This suggests that the highly viscous fluid may actually resist the “leakage” of protein-polysaccharides from cartilage.

Ogston and Phelps (1961) investigated the molecular exclusion properties of hyaluronate, one of the constituents of synovial fluid, and showed that it can effectively resist the diffusion of other macromolecules into solutions containing hyaluronic acid. This property is dependent upon the ratio between length and radius of the molecule, its charge density and its concentration.
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Because any acidic linear polymer could be expected to behave in this manner it was decided to investigate the effect of gelatin on the diffusion of protein-polysaccharides from cartilage.

MATERIALS AND METHODS

*Cartilage*—Calf knee joints were obtained fresh from the abattoir and the cartilage was shaved off in slices measuring 1×2×0.3 centimetres. The slices were washed in chilled physiological saline.

*Gelatin*—The preparation used had a molecular weight of 8×10⁴ and an isoelectric point of 4.6. A series of gelatin solutions was prepared in 0.05 M phosphate buffered saline, pH = 5.5, in concentrations of 1, 2.5, 5, 10, 20 and 40 per cent W/v. Sodium azide (0.02 per cent W/v) was added to prevent bacterial growth.

Approximately 5 grammes of cartilage were added to 100 millilitres of each solution and stirred at 37 degrees Centigrade for sixteen hours. The solutions were then filtered through Celite supported on Whatman 541 paper and two layers of lint. Aliquots were taken and after precipitation of the gelatin by the addition of perchloric acid, dilutions of twenty, fifty and 100 times were made. Uronic acid concentration was estimated by the method of Bitter and Muir (1962).

A control solution, to which no gelatin had been added, was treated in the same way. Perchloric acid caused no precipitation of the protein-polysaccharide.

Protein-polysaccharide prepared by the method of Malawista and Schubert (1958) was added to the different concentrations of gelatin referred to above and incubated in a shaking water bath at 37 degrees Centigrade for sixteen hours. The gelatin was precipitated as before and uronic acid determinations were performed on the supernate to estimate recovery of protein-polysaccharide. In all cases recovery of more than 90 per cent was obtained.

The incorporation of labelled sulphate is a valid indication of the synthesis of the entire protein-polysaccharide complex (Greer, Janicke and Mankin 1968). Accordingly small blocks of articular cartilage were incubated in a tissue culture medium (three parts Gey’s solution to one part TC 199 supplemented with glucose 1.5 milligrams per millilitre) to which had been added Na₂₃⁵SO₄ (15 μ c/millilitre). After twelve hours the blocks were removed, washed in chilled 2.5 per cent sodium sulphate and the amount of incorporated radioactivity was estimated (Sweet 1970) in order to be sure of uniform labelling of protein-polysaccharide. These blocks of cartilage were then subjected to the same extraction process, both in plain buffer and in the presence of varying concentrations of gelatin. The amount of ₃⁵S-protein-polysaccharide in the supernate after precipitation of gelatin was determined by liquid scintillation spectrometry in a Beckman model LS 133 liquid scintillation spectrometer. A PPO-Dimethyl POPOP cocktail was used and counts per minute were determined in two channels—a ¹⁴carbon window and a ³²phosphorus window. The cartilage slices were sectioned at 5 microns in an international cryostat and stained with toluidine blue.

**RESULTS**

The relationships between the concentration of gelatin used and the amounts of uronic acid-containing material (chondroitin sulphate) or ³⁵S labelled material (protein-polysaccharide) are identical (Fig. 2). The weaker (5 per cent)
concentrations of gelatin differed little from the control solution in their effect upon protein-polysaccharide loss from cartilage. At a gelatin concentration of 40 per cent, however, there was complete inhibition of protein-polysaccharide diffusion from the tissue.

### FIG. 3
Sections of cartilage stirred in 40 per cent gelatin solution, stained with toluidine blue. There is some loss of metachromatic staining material in the centre of the specimen but an accumulation of material around the edges. (×160.)

### FIG. 4
Sections of cartilage stirred in 0.05 M phosphate buffered saline, pH = 7.3 at 37 degrees Centigrade for sixteen hours, stained with toluidine blue. There is uniform complete loss of metachromasia. (×160.)

Sections of the cartilage that had been stirred in the 40 per cent gelatin solution showed some loss of metachromasia in the centre of the specimen but intense staining around the edges (Fig. 3). This contrasted markedly with the sections of cartilage stirred in the control solution, where there was marked and uniform loss of metachromasia (Fig. 4).
DISCUSSION

Three salient facts have been established by these experiments. Firstly, protein-polysaccharide is easily lost from cartilage which is stirred in buffered salt solution. Secondly, the diffusion of the molecules into the surrounding medium can be prevented by high concentrations of gelatin. And thirdly, the gelatin solution does not prevent the movement of protein-polysaccharides within cartilage. It does, however, appear to retard the loss of these molecules into the surrounding medium (Figs. 3 and 4).

Ali (1964) and Dziewiatkowski, Tourtellotte and Campo (1968) showed that protein-polysaccharides can be broken down by the hydrolytic enzymes present in cartilage. Ali further maintained that subsequent release of the protein-polysaccharides from cartilage could be inhibited to a certain extent by the addition of arginine or one of its analogues to the buffer medium. Hoffman's method of extracting protein-polysaccharides from cartilage undoubtedly relies on the action of the enzyme with catheptic activity described by Ali, to disrupt the basic peptide bridges, so causing disaggregation of the larger molecules referred to above. The resulting monomers will readily diffuse into a suitable aqueous medium.

The experiments described here show that this diffusion can be retarded by the addition of a suitable linear polymer to the medium. Likewise, the loss of ground substance from normal articular cartilage in vivo must be inhibited, at least in part, by the exclusion property of hyaluronate in synovial fluid. The molecular weight of hyaluronate is approximately fifty times greater than that of the gelatin preparation used. It is not surprising, therefore, that synovial fluid, which contains hyaluronate in a concentration of about 1 per cent, appears to exert an excluding effect of the same order as that of 40 per cent gelatin solution.

Clearly a satisfactory balance between the synthesis and loss of cartilage matrix is dependent upon several factors. The size of the protein-polysaccharide molecule would, in itself, prevent its diffusion out of cartilage matrix. In addition the exclusion effect of hyaluronate in synovial fluid offers a further resistance to such diffusion. A further possibility, of course, is that much of the available water could be bound by high concentrations of gelatin or hyaluronate. It is suggested that for cartilage breakdown to occur it is necessary, 1) to bring about disaggregation of the large protein-polysaccharide molecules (for instance, by enzymatic action); and 2) to reduce the exclusion effect of hyaluronate, either by dilution of the synovial fluid or by enzymatic degradation of the hyaluronate itself.

SUMMARY

1. The hyaluronate of synovial fluid is an acidic linear polymer which can effectively resist the diffusion of other macromolecules into its domain. Gelatin was used as an experimental model for hyaluronate, to investigate its effect upon the diffusion of protein-polysaccharides from cartilage slices.

2. The concentration of protein-polysaccharide in the extracting medium was quantitated by uronic acid estimation and liquid scintillation spectrometry of $^{35}$S-sulphate-labelled protein-polysaccharide.

3. Concentrations of gelatin in excess of 20 per cent (W/v) significantly retarded diffusion of protein-polysaccharides out of cartilage slices, but evidently not the movement of these molecules within cartilage.

4. It is suggested that disaggregation of both the protein-polysaccharide molecules of cartilage and the hyaluronate of synovial fluid contribute to cartilage breakdown.

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REFERENCES


