Isolation of Human Synovial-Fluid Hyaluronate by Density-Gradient Ultracentrifugation and Evaluation of its Protein Content

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1. A compound of hyaluronate and protein, called hyaluronate–protein was isolated from pooled human synovial fluids by caesium chloride density-gradient ultracentrifugation. 2. The isolated hyaluronate–protein was labelled with [^{125}I]iodide and the following studies were done. (a) Ultracentrifugation in caesium chloride showed that the protein moiety (^{125}I counts) and hyaluronate (hexuronate) sedimented together in the middle of the gradient. (b) The labelled hyaluronate–protein was treated with trypsin, and ultracentrifugation showed that peptide fragments (^{125}I counts) were dispersed throughout the gradient, indicating proteolytic digestion. Hyaluronate sedimented in the middle of the gradient. (c) The labelled hyaluronate–protein was digested with streptococcal hyaluronidase, and ultracentrifugation showed that hyaluronate fragments were dispersed throughout the gradient, indicating digestion of the polysaccharide. The protein moiety, without attached hyaluronate, now sedimented at the top of the gradient. (d) Ultracentrifugation of labelled hyaluronate–protein in 4M-guanidinium chloride showed that protein and hyaluronate sedimented together. 3. These studies confirm that hyaluronate is combined with a small quantity of protein in normal human synovial fluid. A mild method for the rapid isolation of hyaluronate–protein in good yield is described.

Hyaluronate isolated from human or ox synovial fluids by a variety of methods has been reported to contain 4–2% or less protein (Sandson & Hamerman, 1962; Pigman *et al.*, 1961; Silpananta *et al.*, 1968; How *et al.*, 1969), but this low protein content has sometimes been considered to represent contamination. Sandson & Hamerman (1962) reported isolation of hyaluronate from normal human synovial fluids by a combination of chromatography and ultrafiltration by using Millipore filters whose pore diameter (0.22μm) permitted serum proteins to pass but retained hyaluronate. The hyaluronate had a protein content of 2%, and to confirm that the protein and hyaluronate were firmly combined, the protein was labelled with [^{131}I]iodide. By using zone electrophoresis, it was observed that the hyaluronate (measured by hexuronate analysis) and protein (^{131}I counts) migrated together over the pH range 4.5–10.6, and were not dissociated in 0.2M-NaCl or 6M-urea. The compound of hyaluronate and protein was called hyaluronate–protein.

In the present paper we describe an independent method for the isolation of hyaluronate–protein from human synovial fluid by using density-gradient ultracentrifugation. Additional studies with ^{125}I to

label the protein moiety confirm that the hyaluronate and protein are combined.

Methods

Preparation of hyaluronate–protein

Collection of synovial fluid. Synovial fluid was obtained by arthrocentesis of apparently normal knee joints obtained *post mortem* from subjects less than 60 years old. Protein concentration was determined by the Folin technique (see below) on each sample and was less than 20mg/ml in each fluid used. Fluids were stored at 5°C and used within 1 week.

Isolation of hyaluronate–protein by ultracentrifugation. About 30ml of pooled synovial fluid was centrifuged at 10000g for 10min at 5°C to remove any cellular debris and was then dialysed at 5°C against three 1-litre changes of 0.05M-potassium acetate, pH7.1. The fluid was diluted 1:2 with 0.05M-potassium acetate, then adjusted to a density of 1.64g/ml by adding 1.13g of CsCl/ml (Gallard Schlesinger Chemical Corp., New York, N.Y., U.S.A.; special biochemical grade), and spun at 100000g for 44h at 5°C in a fixed-angle 40 rotor (Beckman–Spinco model L-2 ultracentrifuge). Twelve 1ml fractions were collected from the bottom of the

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tubes, dialysed at 5°C against three 1-litre changes of 0.05 M-potassium acetate, and those fractions containing hyaluronate–protein were subjected to a second 44 h centrifugation under the same conditions as the first. The fractions from the second centrifugation were dialysed against 0.05 M-potassium acetate as described above and those containing hyaluronate–protein were pooled.

Other methods

Protein and hexuronate analyses. Protein was determined by a modified Folin method (Lowry *et al.*, 1951) with a bovine serum albumin solution as standard. Hexuronic acid determinations were done by a modification (Bowness, 1957) of the carbazole method of Dische (1947).

Labelling of hyaluronate–protein with ^{125}I. A solution of approx. 4 mg of hyaluronate–protein in 2 ml of 0.01 M-tris–HCl buffer, pH 8.0, was iodinated with [^{125}I]iodide by the method of Sandson & Hamerman (1962). The reaction mixture was dialysed against 0.05 M-potassium acetate to remove free iodide. Isolation of the iodinated hyaluronate–protein was then accomplished by density-gradient ultracentrifugation under the conditions described above. ^{125}I radioactivity was determined in a deep-well gamma counter.

Determination of the amino acid labelled with ^{125}I. Labelled hyaluronate–protein (100000 c.p.m. in 2 ml of 0.05 M-potassium acetate) isolated after ultracentrifugation was incubated overnight at 37°C with 2 mg of streptococcal hyaluronidase (Wyeth, Philadelphia, Pa., U.S.A.). The digested hyaluronate–protein was then applied to a Sephadex G-50 (medium grade) column (1.3 cm × 100 cm) washed with 0.05 M-potassium acetate. The excluded peak representing the labelled glycoprotein moiety was pooled and dialysed against 0.04 M-tris–HCl buffer, pH 7.8, containing 0.15 M-NaCl with 0.01 M-propylthiouracil. After dialysis, 2 mg of Pronase (Calbiochem, Los Angeles, Calif., U.S.A.) was added and the mixture was incubated overnight at 37°C. This material was applied to a 2.5 ml column of the quaternary ammonium form of Ag 1 resin (X2, 200–400 mesh) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). The salts were eluted with 10 ml of 0.1 M-ammonium acetate, pH 9, and then the amino acids were eluted with 10 ml of 0.1 M-ammonium acetate, pH 3.4. This material was concentrated by freeze-drying to 0.1 ml and applied to Whatman 3MM chromatography paper (15 cm × 50 cm). Monoiodotyrosine and di-iodotyrosine (50 μg of each) were also applied to the paper, which was then subjected to chromatography in a descending system with butan-1-ol–acetic acid–water (12:3:5, by vol.) as the solvent. Location of the ^{125}I label was determined by radioautography and by cutting the chromatograph into 1 cm strips and count-

ing the radioactivity of each strip in the deep-well gamma counter. The mono- and di-iodotyrosine were detected by staining with diazotized sulphanilic acid (Gross & Leblond, 1951).

Digestion of labelled hyaluronate–protein. Labelled hyaluronate–protein (200000 c.p.m. in 1 ml) was dialysed against 0.05 M-tris–HCl buffer, pH 7.4, containing 0.15 M-CaCl₂ and then incubated with 2 mg of trypsin (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) at 37°C overnight. A second sample of labelled hyaluronate–protein (200000 c.p.m. in 1 ml) was digested with streptococcal hyaluronidase as described above. Control tubes, containing hyaluronate–protein without added enzymes, were incubated in a similar manner. Each digest and control was then diluted to 8.5 ml with 0.05 M-potassium acetate, then adjusted to a density of 1.64 g/ml with CsCl and centrifuged as described above. After fractionation of the gradient, samples were dialysed and hexuronate and ^{125}I radioactivity were determined.

Density-gradient centrifugation of hyaluronate–protein in the presence of guanidinium chloride. Labelled hyaluronate–protein (approx. 300000 c.p.m. in 2 ml) was centrifuged in the presence of 4 M-guanidinium chloride (Schwarz–Mann, New York, N.Y., U.S.A.; Ultra Pure), with 0.597 g of CsCl/ml of solution (final density 1.71 g/ml). After fractionation, the samples were dialysed and analysed as described above.

Results

Isolation of hyaluronate–protein by density-gradient ultracentrifugation

The hyaluronate–protein was located by hexuronate analysis and in each ultracentrifugation was confined to the middle of the gradient at a density of about 1.64 g/ml (Figs. 1 and 2). Serum proteins sedimented at the top of the gradient, and an unidentified component, possibly chondroitin sulphate–protein complex, was present at the bottom after the first ultracentrifugation. Hyaluronate–protein was isolated in good yield with a protein content that varied slightly between 1.4–3.6% (Table 1). This may reflect traces of extraneous protein, or reproducible results, within the limit of the Folin method to detect such small quantities of protein. The component at the bottom was no longer evident after the second ultracentrifugation (Fig. 2). A third ultracentrifugation performed on a portion of samples 1 and 2 (Table 1), did not change the protein content of hyaluronate–protein, and the protein peak at the top of the gradient was no longer evident. The method used for hexuronate analysis is sufficiently sensitive to locate hyaluronate–protein in the fractions of the gradient, but a colorimetric method is not able to
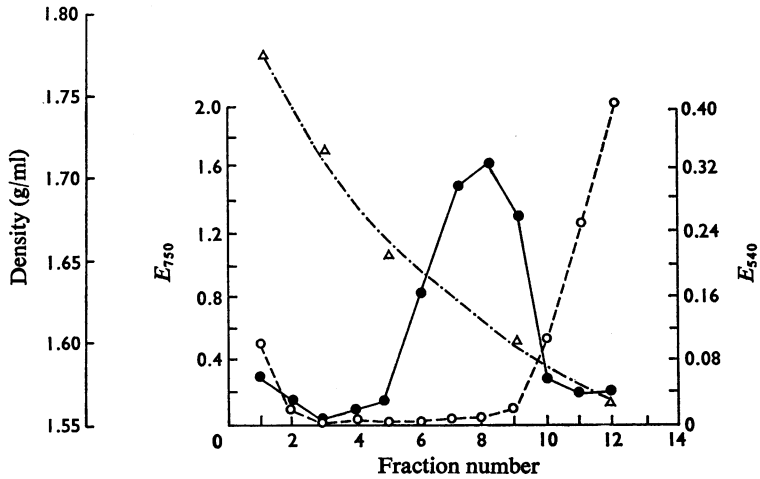
Fig. 1. *Density-gradient ultracentrifugation of synovial fluid*

On this and all subsequent graphs of gradients, the bottom fraction is no. 1. The separation of hyaluronate–protein from synovial fluid proteins with lower density, and an unidentified component, possibly chondroitin sulphate–protein complex with higher density, is shown. The hyaluronate–protein sediments at a density of 1.600–1.650 g/ml. Fractions 6–9 were isolated for re-centrifugation. ○, E_{750} (protein); ●, E_{540} (hexuronic acid); △, density.
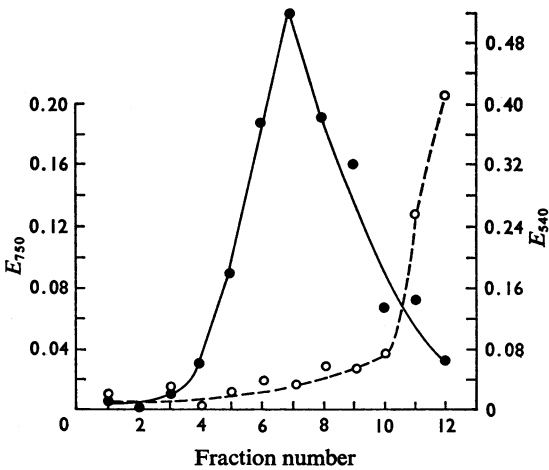
Fig. 2. *Second density-gradient ultracentrifugation of isolated hyaluronate–protein*

The material with a density greater than hyaluronate–protein is no longer obtained at the bottom of the gradient. Fractions 5–9 were isolated and used for further studies. ○, E_{750}; ●, E_{540}.

Table 1. *Isolation of hyaluronate–protein from human synovial fluid by two density-gradient ultracentrifugations in caesium chloride*

For experimental details see the text.

Sample no.	Amount of hyaluronate–protein (mg)		Recovery (%)	Protein content (%)
	At start	Recovered		
1	6.6	4.3	65.0	1.4
2	64.0	36.0	56.0	2.8
3	—	—	—	3.2
4	—	—	—	1.8
5	—	—	—	3.6

Sedimentation of labelled protein

The polysaccharide moiety of hyaluronate–protein (determined by hexuronate analysis) and protein (^{125}I counts), sedimented together during CsCl density-gradient ultracentrifugation (Fig. 3). A discrepancy of one fraction between the hyaluronate and protein was observed consistently. This difference may be explained by assuming that there is some variation of the hyaluronate/protein ratio among hyaluronate–protein molecules. Those with a higher proportion of protein will have a lower density and

detect the small quantity of protein. Therefore the isolated hyaluronate–protein was labelled with ^{125}I, which provided a sensitive method to detect protein.
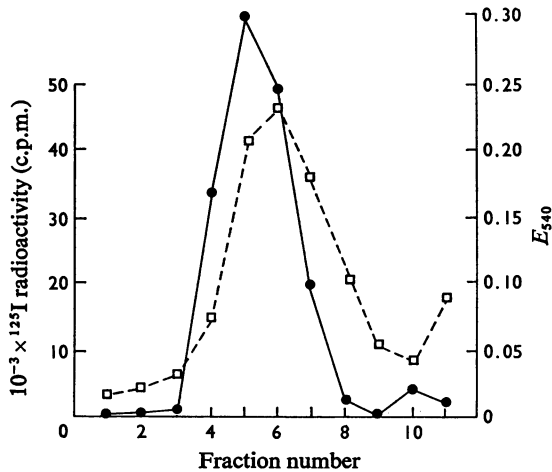
Fig. 3. *Density-gradient ultracentrifugation of hyaluronate–protein after labelling with* [125]*I*

The density of the hyaluronate–protein is not altered by ^{125}I labelling, and the polysaccharide moiety (hexuronate, ●) and protein (^{125}I counts, □) sediment together. (See the text for possible explanation of one tube difference in peaks.)

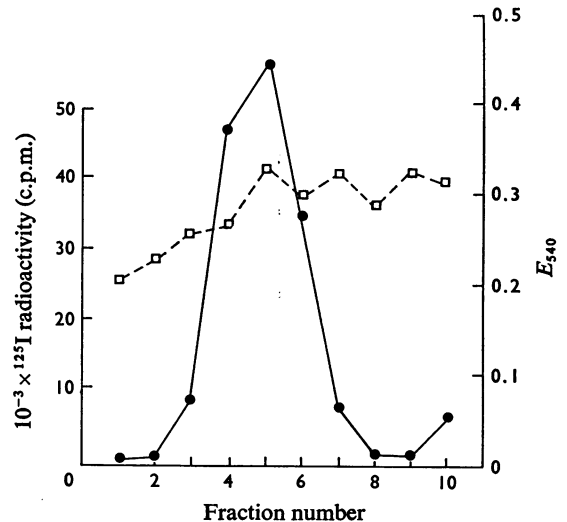will form a zone a little higher in the gradient. Also, assuming that the protein is uniformly labelled, these less-dense molecules will have a higher specific radioactivity on a weight basis.

Trypsin digestion of hyaluronate–protein

After digestion of labelled hyaluronate–protein overnight with trypsin and then ultracentrifugation, the ^{125}I radioactivity was observed to spread throughout the gradient (Fig. 4). There was no change in the sedimentation of the hyaluronate. Peptides released from hyaluronate–protein by trypsin digestion are expected not to form a zone because of their low molecular weights and therefore high diffusion coefficients.

Hyaluronidase digestion of hyaluronate–protein

The results of streptococcal hyaluronidase digestion of labelled hyaluronate–protein established two points (Fig. 5). First, they proved the authenticity of hyaluronate, since fragments of low-molecular-weight polysaccharide failed to form a zone and were scattered throughout the gradient. Secondly, they showed that without the hyaluronate, the protein moiety does not migrate in the middle of the gradient, but rather at the top. If, as would be most unlikely, the protein, because of similar density, sedimented in the
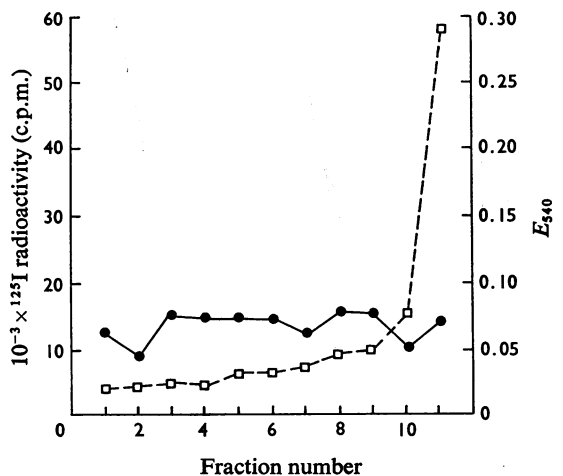

Fig. 4. *Density-gradient ultracentrifugation of trypsin digest of* [125]*I-labelled hyaluronate–protein*

The trypsin released ^{125}I radioactivity which is distributed in all fractions of the gradient. ●, E_{540}; □, ^{125}I radioactivity.

Fig. 5. *Density-gradient ultracentrifugation of streptococcal hyaluronidase digest of* [125]*I-labelled hyaluronate–protein*

The polysaccharide fragments (E_{540}, ●) are distributed evenly throughout the gradient, whereas the protein (^{125}I radioactivity, □) is found at the top of the gradient.

fractions containing the hyaluronate, digestion of the hyaluronate would not alter the sedimentation of the protein, which it clearly does.
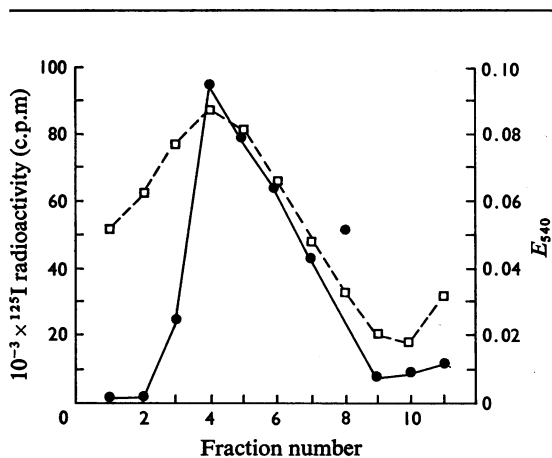

Fig. 6. *Density-gradient ultracentrifugation of* [125]*I-labelled hyaluronate–protein in the presence of* 4M-*guanidinium chloride*

Most of the protein (^{125}I radioactivity, □) remains associated with the polysaccharide (E_{540}, ●).

Ultracentrifugation of hyaluronate–protein in guanidine

To decrease non-covalent interaction between hyaluronate and a contaminating protein, labelled hyaluronate–protein was centrifuged in the presence of 4M-guanidinium chloride. The density of the hyaluronate–protein was altered in guanidine, so that a solution of 1.5mg of hyaluronate–protein in 4M-guanidine sedimented in the middle of the gradient made by adding 0.597g of CsCl/ml of the hyaluronate–protein solution. Most of the protein continues to sediment with the hyaluronate, and the peaks now coincide (Fig. 6). Some protein sediments in a fraction essentially free of hyaluronate, but the nature of this material is not known.

Ultracentrifugation of hyaluronate–protein with [125]*I-labelled albumin*

Although the high concentration and diffuse domain of hyaluronate–protein chains would be likely to exclude non-bound protein, the possibility of entanglement between hyaluronate–protein and serum protein was studied. For this purpose, unlabelled hyaluronate–protein (1.5mg/ml) was mixed with ^{125}I-labelled serum albumin. Ultracentrifugation showed that hyaluronate–protein was in the
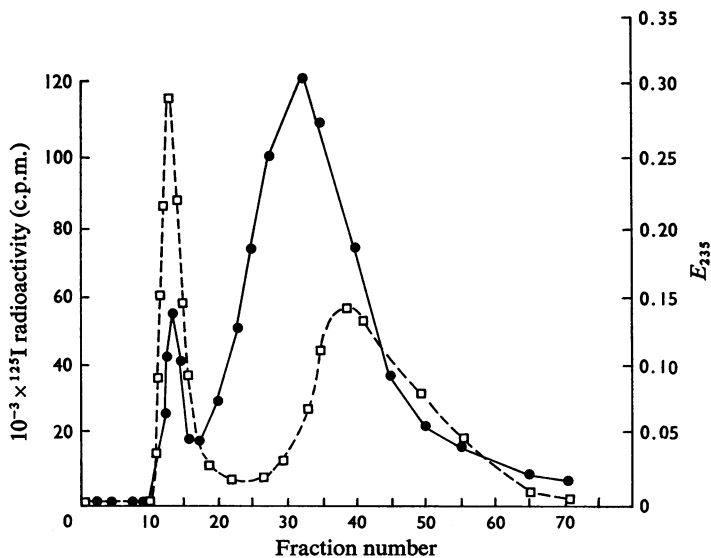

Fig. 7. *Chromatography of* [125]*I-labelled hyaluronate–protein on Sephadex G-50 after digestion with streptococcal hyaluronidase*

Three fractions are observed: the excluded peak (fractions 10–15) containing the glycoprotein; the fragments of digested polysaccharide (fractions 16–50); and low-molecular-weight material containing ^{125}I radioactivity, which may consist largely of non-protein-bound [125]I. □, ^{125}I radioactivity (protein); ●, E_{235} (represents unsaturated disaccharide produced by hyaluronidase digestion of hyaluronate).
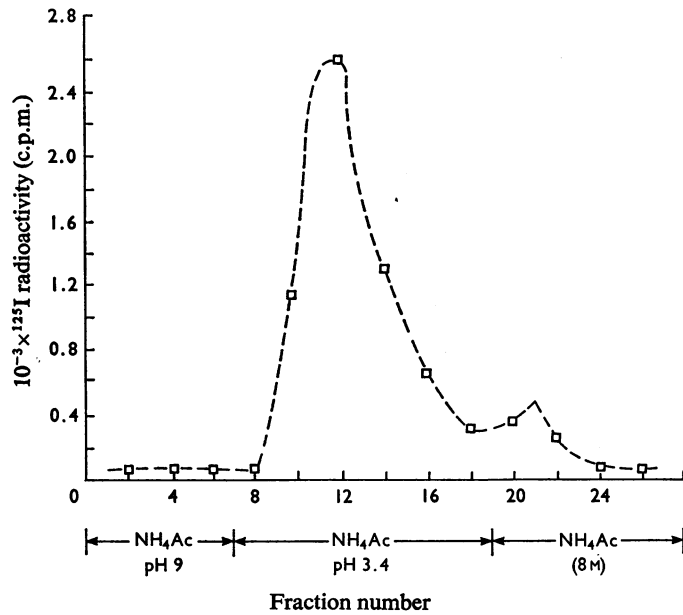
Fig. 8. *Chromatography of the* ^{125}I*-labelled glycopeptide on an AG* 1 *(X2) ion-exchange column after digestion with Pronase*

The labelled amino acids are eluted with 0.1 M-ammonium acetate (NH₄Ac), pH 3.4.

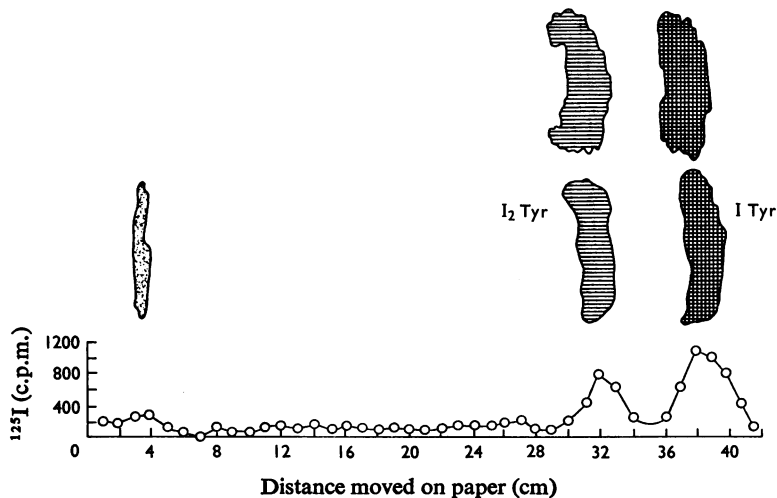Fig. 9. *Paper chromatography of labelled amino acids (Fig. 8)*

Monoiodotyrosine (ITyr) and di-iodotyrosine (I₂Tyr) were used as standards. The labelled amino acids migrated exclusively with the standard amino acids, as determined by staining of the chromatogram, radioautography and cutting the chromatogram into 1 cm strips and counting for radioactivity.

middle of the gradient and the labelled albumin was completely separated at the top.

Identification of the ^{125}I-labelled amino acids

Hyaluronate–protein labelled with ^{125}I was digested with streptococcal hyaluronidase and run through a Sephadex G-50 column (Fig. 7). The excluded peak, which has been shown to consist of the glycoprotein of hyaluronate–protein (Hamerman et al., 1966), contained most of the ^{125}I radioactivity. The included material, which absorbs strongly at 235nm owing to the presence of unsaturated disaccharides, appeared not to be associated with the remaining ^{125}I radioactivity. When the material in the excluded peak was further digested with Pronase and applied to an ion-exchange column, the ^{125}I-labelled amino acids were eluted at a pH where mono- and di-iodotyrosine were known to come off the column (Fig. 8). Free iodide, if present, remains absorbed to the column under the conditions of this study. Counting the radioactivity of the entire column after elution revealed only background counts. Paper chromatography with mono- and di-iodotyrosine as standards, with the ^{125}I-containing material eluted from this column, confirms the association of the ^{125}I with tyrosine in the protein of hyaluronate–protein (Fig. 9).

Discussion

Protein associated with hyaluronate by covalent bonds must form only a minimal part of the hyaluronate–protein molecule, which is composed primarily of macromolecular hyaluronate. Evaluation of the amino acids in hyaluronate–protein is thus very difficult because of the minute quantities of protein present in even large samples of hyaluronate–protein. Hamerman et al. (1966) isolated a glycopeptide from hyaluronate–protein and published an amino acid analysis of this material. Laurent (1970) reported the isolation of rooster comb hyaluronate by using CsCl density-gradient centrifugation. He noted that the amino acid analysis he obtained agreed with that found by Swann (1968) with similar material isolated by cetylpyridinium chloride precipitation and chromatography. Comparison of the amino acid analyses in these reports reveals similarities between the most commonly present amino acids. This supports the concept of a firm association of hyaluronate and a specific protein.

The use of density-gradient ultracentrifugation to isolate hyaluronate–protein overcomes the problem of contamination by protein or protein–polysaccharides. Hyaluronate–protein in solution excludes other macromolecules from its domain (Ogston & Phelps, 1961), and this property would tend to eliminate contaminating protein and protein–polysaccharides from the portion of the gradient containing the hyaluronate–protein. Moreover, the synovial-fluid proteins are of lower density, and sediment at the top of the gradient separately from the hyaluronate–protein. One problem with previously described methods of isolating hyaluronate–protein is possible contamination of it by protein–polysaccharides containing chondroitin sulphate. These protein–polysaccharides have been detected in ox synovial fluid (Silpananta et al., 1967) and in rheumatoid joint fluids (Barker et al., 1966), but there is no information about their presence in normal human synovial fluid. It has been suggested that the protein isolated with hyaluronate could be combined with chondroitin sulphate (Meyer, 1966). By using the characteristic density of hyaluronate to isolate it, one avoids the limitations of these other isolation methods. Chondroitin sulphate is denser than hyaluronate and sediments at the bottom of the gradient when centrifuged under these conditions, as both we and others (Silpananta et al., 1967) have shown.

Density-gradient ultracentrifugation appears to be the most rapid and mildest method yet reported for the isolation of hyaluronate–protein in good yield. This technique independently and rigorously confirms the combination of human synovial fluid hyaluronate with about 2% protein as reported earlier. Labelling of the protein moiety with ^{125}I provides an extremely sensitive method of monitoring the small quantity of protein. The ^{125}I was shown to be incorporated into mono- and di-iodotyrosine in the labelled hyaluronate–protein.

Density-gradient centrifugation of hyaluronate–protein in 4M-guanidine failed to dissociate most of the protein, a finding that strongly indicates covalent linkage between hyaluronate and protein. Nor does the guanidine disperse hyaluronate chains in the gradient, as might occur if chains of hyaluronate were non-covalently bound to protein to form macromolecular aggregates. This appears to be the condition of native protein–polysaccharide complexes isolated by extraction in guanidinium chloride or $MgCl_2$ from bovine nasal cartilage (Sajdera & Hascall, 1969). After density-gradient ultracentrifugation in 4-M guanidine, protein–polysaccharide complexes are reversibly dissociated into proteoglycan subunits and a 'link' glycoprotein (Hascall & Sajdera, 1969).

The physiological role of the small quantity of protein bound to hyaluronate is not known. Unlike the effects on protein–polysaccharide complexes, where proteolytic digestion destroys the viscosity, such treatment of hyaluronate–protein does not decrease the viscosity (Sandson & Hamerman, 1962). Antigenic properties of the hyaluronate–protein appear to reside in the protein moiety, or in the linkage region between protein and hyaluronate (Blau et al., 1965). The relationship of the synthesis of the protein moiety to the polysaccharide is of interest.

With chondroitin sulphate, the synthesis of this polysaccharide stops when synthesis of the protein moiety is prevented by puromycin (De La Haba & Holtzer, 1965; Telser *et al.*, 1965). The effects of protein inhibitors on hyaluronate synthesis depend on the system studied: streptococci continue to make hyaluronate in the presence of puromycin in the medium (Stoolmiller & Dorfman, 1969), whereas synthesis of hyaluronate by human synovial-cell cultures is partly suppressed by cycloheximide or puromycin (Smith & Hamerman, 1968).

It seems clearly established that there is a firm association of a small amount of protein to hyaluronate. By using the technique described in this paper large quantities of hyaluronate–protein may be prepared, enabling isolation and characterization of the linkage region of this proteoglycan.

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