Isolation and Properties of Hyaluronic Acid from Bovine Heart Valves

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1. A soluble extract of bovine heart valves was obtained after the tissue had been pulverized at liquid-nitrogen temperatures in a mill. 2. Hyaluronic acid was isolated from the crude extract by sedimentation equilibrium in a caesium chloride density gradient (Franek & Dunstone, 1966). 3. Analysis of the product indicated that it contained 15% of protein and the molar ratio of glucuronic acid to glucosamine was 1.27. 4. Its physicochemical properties, as determined by light-scattering, viscosity and sedimentation studies, suggested that its molecular size and configuration were similar to those of hyaluronic acid isolated from ox synovial fluid (Preston, Davies & Ogston, 1965).

Earlier studies on the physicochemical behaviour of hyaluronic acid in solution were carried out on material isolated from synovial fluid (Preston, Davies & Ogston, 1965), umbilical cord (Laurent, 1957) and vitreous humour (Laurent, Ryan & Pietruszkiewicz, 1960). These studies indicated that the hyaluronic acid isolated from these various sources differed both in chemical composition and in molecular size. All these sources contain, if any, only a loose network of fine collagen fibres and allow an easy and ready extraction of the mucopolysaccharide. No comparable studies have been carried out on the hyaluronic acid known to be present in the denser types of connective tissue such as skin, heart valves and aorta. These tissues contain coarse collagen fibres and elastin which make the extraction of the native mucopolysaccharide molecules difficult. If the biological role of the hyaluronic acid is to be deduced it is perhaps interesting to contrast the properties of the polymer extracted from a dense tissue with that isolated from one of the softer types.

In previous papers (Lowther, Toole & Meyer, 1967; Preston, 1968; Toole & Lowther, 1968) the identification, extraction and characterization of some mucopolysaccharides from bovine heart valves were reported. In this paper the isolation of hyaluronic acid from the same tissue by very mild procedures is described. In many earlier studies (Scott, 1960; Balazs, 1965) the preparative procedures have included such steps as repeated precipitation of the mucopolysaccharide with cetylpyridinium chloride or with ethanol and removal of proteins by enzymic digestion or passage down Celite columns. In our hands such treatment resulted in a material displaying lower viscosities than that obtained by using our present techniques.

Our present studies indicate that the hyaluronic acid isolated from heart valves forms a complex with protein and has physical parameters very similar to the high-molecular-weight hyaluronic acid isolated by the mild filtration techniques of Preston *et al.* (1965).

EXPERIMENTAL

Extraction of mucopolysaccharides from heart valves. Semilunar and arterioventricular heart valves were dissected from adult steers within 2 hr. of death. The pooled valves (350 g. wet wt.) were washed three times with cold water (500 ml.) and minced to give coarse valve fragments. These fragments were then dropped into liquid nitrogen and the frozen tissue was powdered by passing it through the 20 mesh attachment in a Wiley Intermediate Mill (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.). The powdered tissue was stirred at 4° with an equal volume of 0.2 M-NaCl-0.12 M-NaHCO₃, pH 6.8; chloramphenicol (2 µg./ml.) was added to the buffer to minimize bacterial activity. After 16 hr. the sediment was removed by centrifugation at 28000g for 30 min. in the no. 30 rotor of the Beckman-Spinco model L preparative ultracentrifuge. The residue was re-extracted for a further 16 hr. with an equal volume of the buffer solution and the supernatant fraction again collected by centrifugation. The combined supernatant fractions, containing a mixture of serum proteins and mucopolysaccharides, were dialysed against several changes of 0.05 M-NaCl-0.12 M-NaHCO₃. The precipitate that formed was removed by centrifuging as before, and the lipid material was removed by passing the supernatant fraction through a Postlip filter paper (Evans Adlard Co., Cheltenham, Gloucestershire). The volume of the extract was decreased to about 100 ml. by pressure dialysis.

Fractionation of salt extract by caesium chloride density-gradient centrifugation. The fractionation of the extract was carried out by equilibrium density-gradient centrifugation (Franek & Dunstone, 1966). A preliminary centrifugation in

the no. 50 rotor of the Beckman-Spincor model L preparative ultracentrifuge required 10 ml. of solution (density 1.5 g./ml.) to fill the tube completely. After 40 hr. at 100000g the contents of the tube were divided into four fractions by means of a Beckman tube slicer. Each fraction was assayed for protein and uronic acid; of the total protein present in the tube, 99% was found in the top fraction. Recentrifugation of the combined bottom three fractions under the same conditions, followed by the removal of the top 1 ml. of solution, resulted in the removal of the small amount of free protein still present. Some protein remained associated with the mucopolysaccharides. By the method of Silpananta, Dunstone & Ogston (1967) the separation of the different mucopolysaccharides present was achieved by equilibrium centrifugation of the purified extract at a density of 1.71 g./ml. After 90 hr. at 100000g, the contents of the tube were fractionated by use of the Beckman system (type SW 39) into 13 fractions. Each fraction was assayed for galactosamine, total hexosamine, uronic acid and protein. These studies indicated that separation of the polymeric components had occurred, and in subsequent experiments the contents of the tube were divided into three fractions: (i) 2 ml. containing fraction B (mean density 1.76 g./ml.); (ii) 2 ml. containing fraction M (mean density 1.73 g./ml.); (iii) 5 ml. containing fraction T (mean density 1.69 g./ml.).

To investigate the homogeneity of fraction T, it was centrifuged again at 100000g for 72 hr. at an initial density of 1.66 g./ml. (mucopolysaccharide concentration 1 mg./ml.). Fraction T was dialysed against water to free it from CsCl, and stored at -20° .

Enzymic studies to determine the nature of fraction T. (a) Action of testicular hyaluronidase. Samples of fraction T were incubated with testicular hyaluronidase (Rhodase; Evans Medical Ltd., Speke, Liverpool; 1500 i.u./mg. of mucopolysaccharide, in 0.1 M-sodium-acetate-0.1 M-NaCl, pH 5.0) at 37° for 1.5 hr. Hyaluronate isolated from bovine vitreous humour and chondroitin sulphate from bovine cartilage were used as controls. The degradation of the mucopolysaccharide was followed by the turbidimetric method of Moretti & Whitehouse (1963), with rivanol as precipitant.

(b) Action of proteolytic enzymes. Samples of fraction T were incubated with Pronase (B grade, lot 63807; Calbiochem, Los Angeles, Calif., U.S.A.) in 0.5 M-KH₂PO₄ adjusted to pH 7.5 with 2 M-NaOH at 37° for 2 hr. After digestion the solution was dialysed against several changes of 0.1 M-NaCl-0.12 M-NaHCO₃, and the reduced viscosity was measured in a capillary viscometer. The mucopolysaccharide was isolated by the precipitation technique with cetyltrimethylammonium bromide (Lowther *et al.* 1967) and the protein and uronic acid content measured.

Reagents. Analytical-grade CsCl was used in all experiments. All other reagents used were of analytical quality, unless otherwise specified.

Solution preparation. Solid CsCl was added to the appropriate solutions to give the required densities, which were checked by using a glass syringe (Hamilton Co., Whittier, Calif., U.S.A.) as a pycnometer.

Analytical methods

Uronic acid. This was determined by the carbazole method of Bitter & Muir (1962) with D-glucuronic acid

(Matheson, Coleman & Bell, East Rutherford, N.J., U.S.A.) as standard.

Hexosamine. Samples were hydrolysed with 4 M-HCl at 100° for 16 hr. Total hexosamine was determined by the method of Cessi & Piliego (1960) and galactosamine was determined by the method of Cessi & Serafini-Cessi (1963). D-Glucosamine hydrochloride (Hopkin and Williams, Chadwell Heath, Essex) and D-galactosamine hydrochloride (Mann Research Laboratories, New York, N.Y., U.S.A.) were used as standards.

Protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin (Commonwealth Serum Laboratories, Melbourne, Vic., Australia) as standard. As interference with this reaction was noticed when high concentrations of CsCl were present, the salt was removed by dialysis against 0.12 M-NaHCO₃ solution, pH 6.8, before the determination was carried out.

Sulphate. This was determined by the method of Antonopoulos (1962) with (NH₄)₂SO₄ as a standard.

Physical methods

All measurements were made on solutions of fraction T in 0.1 M-NaCl-0.12 M-NaHCO₃ buffer, pH 6.8. Each sample was dialysed for 2 days against several changes of buffer before the experiment.

Viscosity. Viscosities were measured at 20° either in an Ostwald capillary viscometer (BS/U type B, water time 50 sec.) or in a Zimm rotating-cylinder viscometer (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.).

Reduced viscosities of the fraction T solutions were calculated by comparison of flow times (in the capillary viscometer) or revolution times (in the Zimm viscometer) with those obtained with solvent.

Light-scattering. This was measured in a Sofica (Le Mesnil, Saint Denis, Seine-et-Oise, France) photometer between 30° and 150° with unpolarized light of wavelength 436 nm., as described by Preston *et al.* (1965).

Sedimentation. This measurement was performed in a Beckman-Spincor model E ultracentrifuge equipped with schlieren optics. Velocity sedimentation was observed at 50740 rev./min. at 20° in cells with 0.3 cm. or 1.2 cm. aluminium centrepieces. The sedimentation behaviour of fraction T over a range of concentrations (1.1×10^{-4} – 9.0×10^{-3} g./ml.) was studied. Equilibrium sedimentation was carried out as described by Nichol, Ogston & Preston (1967). Estimates of the non-ideality coefficients α_1^* and α_2^* were made by rearranging eqn. 4 of Nichol *et al.* (1967) into the form:

$$\frac{\omega^2(\partial\rho/\partial c)rc}{RT\partial c/\partial r} - \frac{1}{M} = \alpha_1^*c + 2\alpha_2^*c^2$$

from which α_1^* and α_2^* were estimated by least squares by using the method of Fletcher & Powell (1963) programmed for a CDC 3200 digital computer to perform the minimization. The density increment of fraction T was taken to be 0.31.

RESULTS

Separation of fractions

Fig. 1 gives the results of determination of galactosamine and total hexosamine in the density gradient of initial density 1.71 g./ml. The results

are expressed on the assumption that the sample was a mixture of hyaluronic acid and chondroitin sulphate. The chondroitin sulphate was determined by the galactosamine assay and the hyaluronic acid content was calculated by subtracting the galactosamine content from the total hexosamine content. The top 5 ml. of the tube (fraction T) contained most of the material thought to be hyaluronic acid. The result of recentrifuging fraction T at an initial density of 1.66 g./ml. is given in Fig. 2, where the distribution of uronic acid throughout the tube is shown. Except for the

material at the extreme top and bottom of the tube, the ratio of protein to uronic acid was constant throughout and corresponded to a protein content of $14.9 \pm 1.5\%$. This constant protein composition suggests that the material was present as a mucopolysaccharide-protein complex.

Composition and the nature of fraction T

The chemical analysis of the material indicated that it contained glucosamine (free base) 26%, galactosamine (free base) 2.5%, uronic acid (free acid) 40.2%, sulphate 1.2% and protein (Lowry *et al.* 1951) 14.9%. All values are expressed in terms of the dry weight of the substance. Assuming that all the hexosamine is *N*-acetylated and allowing for the cation equivalence (as Na^+) of all the negatively charged groups, the analytical values account for a recovery of 90% of the dried material.

The action of hyaluronidase on fraction T showed that 98% of the material was digested. This evidence taken in conjunction with the analysis suggested that fraction T was mainly a hyaluronic acid-protein complex. The presence of sulphate and galactosamine indicated that the sample may have contained about 7% of chondroitin sulphate.

The viscosity of a solution of the hyaluronic acid-protein complex was unaffected by digestion with Pronase although the protein content was lowered to 2.4%.

Physical measurements on fraction T

Viscosity. The results of measurements made in the capillary viscometer over a range of concentrations of fraction T (1.0×10^{-4} – 1.5×10^{-3} g./ml.) are plotted in Fig. 3. It is clear that there was a marked effect of salt concentration on the reduced

Fig. 1. Preparative density-gradient separation of aqueous extract of bovine heart valves. The initial density was 1.71 g./ml. and the concentration of mucopolysaccharide approx. 1 mg./ml.; fractions (0–7 ml.) were collected. The hexosamine concentration ($\mu\text{g./ml.}$) (●, ○) and density ($\blacktriangle$) are plotted against volume. The hexosamine concentration is expressed in terms of a mixture of hyaluronic acid (●) and chondroitin sulphate (○) as described in the text. The fractions were pooled into fractions T, M and B as shown.

Fig. 2. Preparative density-gradient centrifugation of fraction T. The initial density was 1.66 g./ml. and the concentration of fraction T approx. 1 mg./ml. The uronic acid concentration ($\mu\text{g./ml.}$) (●) and density ($\blacktriangle$) are plotted against volume.

Fig. 3. Comparison of the reduced viscosities of solutions of fraction T measured in a capillary viscometer at sodium chloride concentrations of 1 mM (○) and 0.1 M (●).

Fig. 4. Reduced viscosities at zero shear stress of solutions of fraction T.

Fig. 5. Variation of the reciprocals of the sedimentation coefficient of fraction T (●) and of hyaluronic acid from ox synovial fluid (○) with concentration. The values for the ox synovial-fluid preparation are taken from the data of Preston *et al.* (1965).

viscosity. Because solutions of fraction T exhibited non-Newtonian behaviour, a limited series of measurements were made in a Zimm rotating-cylinder viscometer (Zimm & Crothers, 1962). The measurements were made in 0.1 M-sodium chloride–0.12 M-sodium hydrogen carbonate at shear stresses in the range 1.32×10^{-3} – 13.38×10^{-3} dynes/cm.² over a range of concentrations of mucopolysaccharide (0.15×10^{-3} – 1.08×10^{-3} g./ml.). Over this range the reduced viscosity was independent of shear stress. In Fig. 4 the reduced viscosities, obtained by extrapolation to zero shear stress, are plotted against concentration and yield an intrinsic viscosity of about 3300 ml./g.

Sedimentation velocity. The sedimentation behaviour of fraction T is shown in Fig. 5. The results of Preston *et al.* (1965) on hyaluronic acid isolated from ox synovial fluid are included in this plot.

Fig. 6. Schlieren diagram of the equilibrium sedimentation in 0.1 M-NaCl–0.12 M-NaHCO₃ of fraction T (conditions: 21740 rev./min. for 30 hr.; 1.2 cm. $2\frac{1}{2}^\circ$ filled Epon double-sector cell; 20°; initial concentration, 1.50 mg./ml.; schlieren angle, 70°).

There was a close similarity in the behaviour of the two materials, although the sedimentation coefficients of fraction T were consistently less at the same total concentration.

Equilibrium sedimentation. The equilibrium-sedimentation curve is shown in Fig. 6. The character of the curve is similar to that of the hyaluronic acid preparation of Nichol *et al.* (1967). The rise of the gradient of concentration at the bottom of cell could be due to the chondroitin sulphate shown to be present (cf. Silpananta *et al.* 1967). Estimates of the non-ideality coefficients α_1^* and α_2^* were 7.1×10^{-3} and 3.8×10^{-3} respectively and compare favourably with those found by Nichol *et al.* (1967).

Light-scattering. The results were calculated with the use of the specific refractive increment of 0.150 measured against equilibrium diffusate (Casassa & Eisenberg, 1964) and extrapolated by the method of Zimm (1948) to give estimates of the molecular weight (12×10^6) and of the radius of gyration (270 nm.). These values are similar to those found by Preston *et al.* (1965) for material isolated from ox synovial fluid.

DISCUSSION

The fractionation of aqueous extracts of bovine heart valves by sedimentation equilibrium in a caesium chloride density gradient appears to be an effective method for separating the hyaluronic acid from the chondroitin sulphate and protein components present in the extract. A preliminary centrifugation at a density of 1.50 g./ml. removed nearly all protein that was not bound to high-polymeric carbohydrate material. After a second centrifugation at a higher density (1.71 g./ml.) the contents of the tube could be divided into three fractions; a clear separation into the top and bottom fractions occurred but the middle fraction was of a complex nature, containing marked amounts of both hyaluronic acid and chondroitin sulphate. Inability to separate these components by subsequent recentrifugation of this fraction indicated that the chondroitin sulphate may be linked to varying amounts of protein. Such findings have been reported by Rosenberg, Schubert & Sandson (1967) for material isolated from bovine nucleus pulposus and for material from bovine nasal cartilage (Franek & Dunstone, 1967). The chemical analysis of the top fraction (T) indicated that it contained a hyaluronic acid-protein complex, and this was subsequently confirmed by the degradative action of hyaluronidase. The molar ratio of glucuronic acid to glucosamine was 1.27, significantly greater than unity, in agreement with similar observations of Preston *et al.* (1965) and Silpananta, Dunstone & Ogston (1968) on hyaluronic acid isolated from synovial fluid. In contrast with results of comparable studies carried out by Silpananta *et al.* (1968), the protein present seemed to be linked strongly to the hyaluronic acid, as a banding of both components occurred at a similar density of 1.65 g./ml. in the gradient. However, the lack of any effect of the enzymic removal of the protein on the viscosity of solutions of this material indicates that the protein does not seem to be involved in maintaining the physical integrity of the complex.

The physicochemical properties of heart-valve hyaluronic acid are very similar to those of samples isolated by Preston *et al.* (1965) from different sources. The molecular weight, size as judged by the radius of gyration, viscosity and sedimentation properties and the thermodynamic non-ideality coefficients are of comparable values. The isolation of this high-molecular-weight hyaluronic acid suggests that the experimental procedures, which involve the freezing of the valve tissue in liquid nitrogen before mechanical disintegration of the tissue, are mild and ensure a relatively native product.

Since the hyaluronic acid found in the bovine heart valves has physicochemical properties similar to those of that isolated from synovial fluid, it is perhaps appropriate to think of its biological role in the same terms, namely that of a lubricant. The continual displacement of the two zona fibrosa relative to one another during the normal pumping action of the heart would require lubrication, and as has been pointed by McCutchen (1966) the hydrodynamic properties of hyaluronic acid are ideal for this purpose.

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