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Fractionation of a Hyaluronic Acid Preparation in a Density Gradient

THE ISOLATION AND IDENTIFICATION OF A CHONDROITIN SULPHATE

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1. Following the suggestion of Nichol, Ogston & Preston (1967) that hyaluronic acid, prepared by filtration from ox synovial fluid, contains a component of high density, such material has been detected and isolated by equilibrium sedimentation in a density gradient of caesium chloride. 2. This material (fraction III) has been characterized as a chondroitin sulphate–protein complex of average molecular weight about 250000. Its amino acid pattern is characteristic of such complexes present in cartilage. It contains a proportion of glucosamine (as well as galactosamine); this is not due to contamination with hyaluronic acid. 3. Preliminary findings on fraction I (low density) and fraction II (intermediate density) suggest that these consist chiefly of protein and hyaluronic acid respectively.

From the form of the equilibrium sedimentation distribution of hyaluronic acid prepared from ox synovial fluid by filtration (Preston, Davies & Ogston, 1965), Nichol, Ogston & Preston (1967) concluded that this material may contain a fraction with a higher density than the main bulk of the material. Because of this possibility, the hyaluronic acid preparation (referred to as UFR*) was examined by equilibrium sedimentation in caesium chloride density gradients in the analytical ultracentrifuge (Meselson, Stahl & Vinograd, 1957; Vinograd, 1963). The material separated into three distinct fractions, with apparent densities less than 1·58g./ml. (fraction I), 1·65–1·66g./ml. (fraction II) and greater than 1·72g./ml. (fraction III) respectively. Equilibrium density-gradient sedimentation was therefore performed on a preparative scale (Franek & Dunstone, 1966), the three fractions being cleanly separated. The present paper describes the isolation of fraction III and its identification as a chondroitin sulphate–protein complex. Barker, Hawkins & Hewins (1966) have recently reported the detection of chondroitin sulphate in some specimens of pathological human synovial fluid.

EXPERIMENTAL

Materials and solutions

Hyaluronic acid. UFR was prepared from normal pooled ox synovial fluids as described by Preston *et al.* (1965).

* Abbreviation: UFR, ultrafilter residue.

Caesium chloride. Analytical reagent grade quality was used for experiments in the analytical ultracentrifuge, and laboratory reagent grade material for preparative experiments.

Solution preparation. The UFR was dialysed against 0·12mM-$NaHCO_3$; dilute solutions were concentrated, when required, by pressure filtration-dialysis in washed cellophan tubes. Solid CsCl was dissolved in the solutions to give the required densities. For examination in the analytical ultracentrifuge, solutions containing CsCl were dialysed to equilibrium against CsCl solution of the same density. The densities of all solutions were checked by using 0·2ml. constriction pipettes as pycnometers.

Concentrations of solutions. These were determined refractometrically with use of the results of Preston *et al.* (1965), or by drying solutions, dialysed against water, to constant weight at 105° in air. Refractive increments of solutions against diffusates were measured in the differential refractometer of Cecil & Ogston (1951) at 546mμ. All weighings were performed in a Mettler single-pan balance sensitive to 1μg.

Analytical methods

Uronic acid. This was determined by the carbazole method (Dische, 1947) with D-glucuronic acid (Sigma Chemical Co., St Louis, Mo., U.S.A.) as standard.

Hexosamine. Preparations were hydrolysed with 4N-HCl at 100° for 4hr. Chondroitin sulphate preparations gave values some 8% higher when 8hr. hydrolysis was used. As analyses of hyaluronic acid-containing fractions were often carried out concurrently, the shorter period of hydrolysis was preferred. Total hexosamine was determined by the Elson & Morgan (1933) procedure as modified by Cessi & Piliego (1960), and galactosamine was determined by the method of Cessi & Serafini-Cessi (1963). D-Glucosamine hydrochloride and D-galactosamine hydrochloride (both

from Sigma Chemical Co.) were used as standards. Both of these compounds were at least 97% pure as measured by the ninhydrin method (Moore, Spackman & Stein, 1958) with L-alanine (puriss, CHR grade; Fluka A.-G., Buchs SG, Switzerland) as standard.

Sulphate. This was determined by the turbidimetric method of Dodgson & Price (1962).

Protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin (Sigma Chemical Co.) as standard.

Amino acids. Preparations were hydrolysed in 6N-HCl at 110° for 22hr. The HCl was removed *in vacuo* and the hydrolysates were redissolved and run on a Beckman amino acid analyser by Dr D. C. Shaw. No corrections were made for the destruction of certain amino acids that often occurs during acid hydrolysis in the presence of carbohydrate.

Infrared spectra. These were measured by Dr E. Spinner with a Perkin–Elmer model 621 dual-grating instrument. Samples (1mg.) were prepared in KBr disks.

Sedimentation

Analytical sedimentation in density gradients. This was performed in a Beckman–Spinco model E ultracentrifuge, in 12mm. cells, with either single-sector (Kel–F; Minnesota Mining and Manufacturing Co.) or double-sector (aluminium-filled Epon; Shell Chemical Co.) centre-pieces. When the double-sector centre-piece was used, one sector contained the solution, the other equilibrium diffusate, care being taken to fill the two sectors identically. A bottom layer of Kel–F fluid approx. 1mm. deep was used. The speed was usually 44770rev./min. The temperature was controlled near 20°. The sedimentation was observed with schlieren optics. The time required to reach equilibrium was about 36hr. with a fluid column approx. 1cm. high. The initial concentration of the material was about 1mg./ml. The equilibrium density-gradient distributions were calculated from the data of Ifft, Voet & Vinograd (1961), and checked by measurement of the schlieren photographs as described by these same authors.

Preparative sedimentation in density gradients. From the time taken to establish equilibrium in the analytical experiments, with a column approx. 1cm. high at a speed of 44770rev./min., it appeared that something like 35 days would be needed to establish equilibrium in the swing-out SW25.2 rotor (Beckman–Spinco model L2 preparative ultracentrifuge) at its highest permitted speed. It seemed possible, however, that satisfactory resolution could be obtained in a much shorter time in an angle rotor. In a preliminary experiment the no. 40 rotor (26° angle of tube) was used with 9ml. of solution (1mg. of UFR/ml., density 1·67g./ml.) overlayed with paraffin oil to fill the tube completely. At the end of 7 days at 31000rev./min. the contents of the tubes were divided into fractions by means of a Beckman tube slicer; estimations of uronic acid in the fractions showed that there were substantial amounts in the top, centre and bottom layers, with little or none in intermediate layers. Measurement of E_{280} (Beckman model DU spectrophotometer) showed a high value for fraction I and very low values for fractions II and III. The density-gradient distributions obtained by measurement of the density of the several fractions agreed closely with the calculated and published values (Ifft *et al.* 1961). In subsequent experiments the no. 50 rotor (20° angle of tube) was used at 42000rev./min.; the tubes were completely filled with UFR solution (approx. 2–3mg./ml.) containing CsCl to give an initial density of 1·67g./ml. After 6 days at 20° the contents were expelled, dropwise, from a hole punched in the bottom of each tube, by forcing kerosene in through the hole in the tube cap by means of a micrometer syringe. The volumes occupied by each fraction were established by analysis of sample tubes for uronic acid. As the fractions were expelled, the following volumes were collected from each tube: (i) 1·5ml. containing fraction III; (ii) 1·6ml. rejected; (iii) 3·9ml. containing fraction II; (iv) 1·1ml. rejected; (v) 1·5–1·9ml. (the volume remaining) containing fraction I. The rejected volumes contained no significant amounts of uronic acid. Fraction III was dialysed free from CsCl against water, pressure-dialysed to a concentration of 0·5–1mg./ml. and stored at −20°.

Analytical sedimentation. Equilibrium sedimentation was performed with Rayleigh optics, in the Beckman six-chamber cell, under conditions suggested by Yphantis (1964) to determine the molecular weight of fraction III. The material was dialysed against 0·2M-NaCl–0·12mM-$NaHCO_3$ before sedimentation; concentrations were 0·97, 0·65 and 0·32mg./ml. The initial part of the run was done at 19160rev./min., which allowed the sedimentation coefficients to be estimated by integration of the Rayleigh patterns at successive times; equilibrium was then established in 8·5hr. at 14290rev./min.

Separate velocity-sedimentation measurements were also made at 50740rev./min., with schlieren optics. The sedimentation coefficients calculated from both sets of results were in satisfactory agreement. In all cases the temperature was controlled near 20°.

RESULTS AND DISCUSSION

Separation of fractions. Fig. 1 shows typical schlieren diagrams obtained in density-gradient sedimentation during approach to equilibrium (Fig. 1*a*) and when equilibrium is fully established (Fig. 1*b*). The fractions are clearly separated under these conditions. Fig. 2 gives the results of estimations of uronic acid in a typical preparative experiment, showing that conditions seen in the analytical cell can be satisfactorily reproduced in a preparative angle rotor. Fig. 1(*c*) shows the analytical equilibrium gradient sedimentation of a sample of isolated fraction III, demonstrating the absence from it of fractions I and II (compare Fig. 1*b*).

It should be pointed out, however, that the displacement of solution from the preparative tubes does not, under these conditions, give a satisfactory recovery of fraction I. Table 1 shows that, although the recoveries of uronic acid and hexosamine are satisfactory, only about 20–25% of the original total protein was recovered. Presumably fraction I, consisting largely of protein, was concentrated in a very small volume at the top of the tube, and this was not completely displaced from the tube by the kerosene. In this paper we are, however, concerned only with fraction III.

The period chosen for establishing equilibrium in

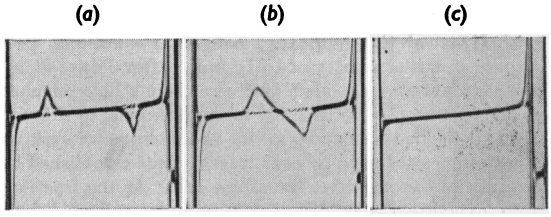

Fig. 1. Density-gradient schlieren patterns of UFR and fraction III. Details of the experimental procedures are given in the text. The speed of centrifuging was 44770 rev./min. and the schlieren phase-plate angle was 60°. (*a*) UFR; concn. 1·1 mg./ml.; initial density 1·66 g./ml.; 21 hr. after reaching speed. (*b*) UFR; same experiment as (*a*); 69 hr. after reaching speed; fraction I has accumulated at the upper meniscus, fraction II in the middle of the cell and fraction III at the bottom. (*c*) Fraction III; concn. 0·76 mg./ml.; initial density 1·65 g./ml.; 16 hr. after reaching speed.

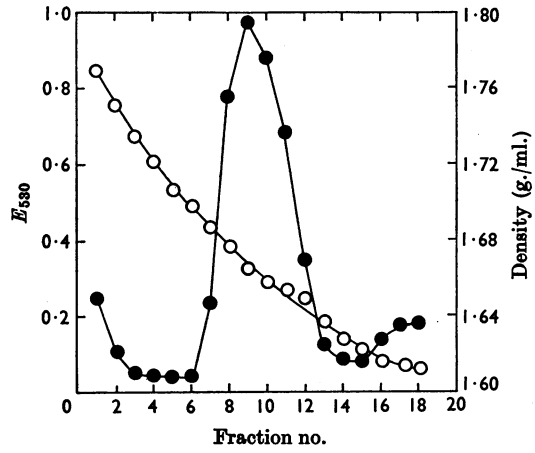

Fig. 2. Preparative density-gradient separation of UFR. Initial density, 1·67 g./ml.; concentration of UFR, approx. 3 mg./ml.; 0·5 ml. fractions were collected as described in the text and are numbered in the order of their collection. The uronic acid extinction (E_{530}) values (●) and the densities (○) are plotted against fraction no.

the preparative experiments was based on the assumption that the effective distance through which sedimentation has to occur is the diameter of the tube (1·6 cm.) and that the mean radius of rotation (in the no. 50 rotor) is 5·4 cm. compared with 6–7 cm. in the analytical rotor. This would predict an equilibration time of about 80 hr. at 42000 rev./min., and this period was approximately doubled to provide a margin of safety.

Composition of fraction III. The analytical results are given in Tables 2 and 3. Taking the proportion

Table 1. *Recovery of material from a preparative density gradient separation of ultrafilter residue*

	Uronic acid (μg.)	Hexosamine (μg.)	Protein (μg.)
Fraction I	233	284	152
Discard fraction I–II*	229	254	53
Fraction II	1618	1622	140
Discard fraction II–III	35	43	29
Fraction III	97	97	78
Total	2212	2300	452
Original UFR	2170	2168	2100

* Owing to slight contamination with fraction II, the hexosamine and uronic acid values are higher than the values predicted from Fig. 2.

Table 2. *Analysis of fraction III*

All values are expressed as g./100 g. dry wt. of substance and are mean values of replicate determinations.

Glucosamine (free base)	3·7
Galactosamine (free base)	23·5
Uronic acid (free acid)	27·2
Sulphate (SO_4^{2-})	15·8
Protein (Lowry *et al.* 1951)	15·8
Protein (from uncorrected amino acid analysis; Table 3)	12·6

of protein as 15·8%, assuming that all the hexosamine is acetylated and allowing for the cation equivalence (as Na^+) of all negatively charged groups, the analytical values represent a recovery of 91·4% of the dried material. The values for uronic acid, hexosamine and sulphate are close to those reported for chondroitin sulphate–protein complex from other sources (Scheinthal & Schubert, 1963; Muir, 1958). Of the hexosamine, a significant proportion is glucosamine, not the galactosamine characteristic of chondroitin sulphates. The presence of a proportion of glucosamine in chondroitin sulphate–protein complexes is, however, not unusual (Anderson, Hoffman & Meyer, 1965; Rosenberg, Johnson & Schubert, 1965); it may be ascribed to the presence of keratan sulphate. It could not be ascribed to the presence of hyaluronic acid, as material corresponding to this fraction (fraction II) has been shown to be absent (Fig. 1*c*). In this experiment, if the glucosamine of the preparation had been due to the presence of hyaluronic acid, a zone with mean density near 1·65–1·66 g./ml. would have been detected.

The infrared spectrum of a freeze-dried salt-free preparation shows a prominent band at 820 $cm.^{-1}$ and minor bands at 930 $cm.^{-1}$ and 850 $cm.^{-1}$. This suggests that the material contains mainly chondroitin 6-sulphate with some chondroitin 4-sulphate

(Mathews, 1958; Lloyd, Dodgson, Price & Rose, 1961). The band at 1000cm.$^{-1}$ usually associated with chondroitin 6-sulphate was not resolved.

Table 3. *Comparison of the amino acid compositions of fraction III, ultrafilter residue, ultrafilter-residue protein and chondromucoprotein*

No corrections have been made for the destruction of amino acids that occurs when amino acid-containing material is hydrolysed with mineral acid in the presence of carbohydrate. Hydrolysis conditions are described in the text.

	Amino acid composition (moles/100moles of amino acids)			
	Fraction III	UFR*	UFR protein*	Chondro-mucoprotein†
Lys	2·3	5·4	4·8	3·5
His	1·3	1·9	1·6	1·7
Arg	2·2	4·3	3·8	4·5
Asp	5·8	9·3	8·8	8·5
Thr	5·8	8·3	8·6	5·8
Ser	14·1	8·9	10·7	9·2
Glu	15·2	12·2	11·7	12·5
Pro	9·1	7·8	6·9	9·3
Gly	13·8	7·9	7·9	12·7
Ala	6·7	6·8	5·8	7·4
CyS	—	—	1·3	0·7
Val	6·6	8·3	9·0	6·3
Met	Trace	1·0	1·1	0·7
Ile	3·6	3·9	3·6	3·8
Leu	7·9	7·5	7·6	7·8
Tyr	1·3	2·9	3·4	1·9
Phe	4·3	3·6	3·4	3·5

* Calculated from the data of Preston *et al.* (1965).
† From Pal *et al.* (1966).

The protein content of 15·8% (Lowry *et al.* 1951; Table 2) is of the order commonly found in preparations that have not been subjected to proteolysis (Scheinthal & Schubert, 1963; Muir, 1958); this value shows reasonable agreement with the value of 12·6% calculated from the uncorrected amino acid values given in Table 3. By applying corrections (Anderson *et al.* 1965) for the destruction of amino acids that occurs during hydrolysis in the presence of carbohydrate, this value would become about 14%.

The amino acid composition differs in several respects from those reported by Preston *et al.* (1965) for UFR and for the whole protein of UFR. It resembles more closely that of chondroitin sulphate–protein complexes of cartilage (Scheinthal & Schubert, 1963; Anderson *et al.* 1965; Pal, Doganges & Schubert, 1966). The amino acid pattern is characterized by a high proportion, and a near equivalence, of serine, glutamic acid and glycine, with corresponding lower proportions of aspartic acid and threonine.

Molecular weight and configuration of fraction III. Fig. 3 shows plots (Yphantis, 1964) obtained from the Rayleigh interference patterns of the equilibrium sedimentation experiments. It is remarkable that, though each of these plots deviates little from linearity, their slopes differ at different concentrations, indicating average apparent molecular weights that decrease with increasing concentration (Fig. 4). Since each slope was determined over the whole of the distribution, this variation can be due only to a degree of thermodynamic non-ideality. The effect of polydispersity of the material alone would be to give an Yphantis plot concave upwards, whereas non-ideality alone would give a plot convex

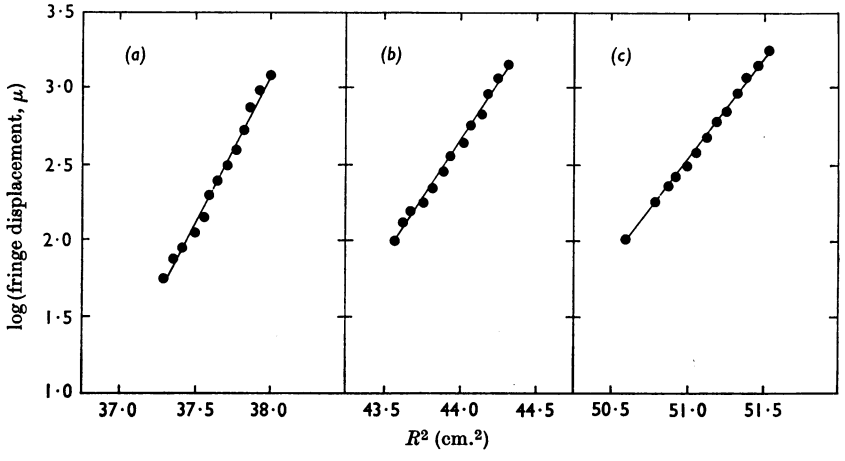

Fig. 3. Equilibrium sedimentation of fraction III. Logarithms of fringe displacements (μ) are plotted against R^2 (R is the distance from centre of rotation in cm.). Concentrations were: (*a*) 0·97mg./ml.; (*b*) 0·65mg./ml.; (*c*) 0·32mg./ml.

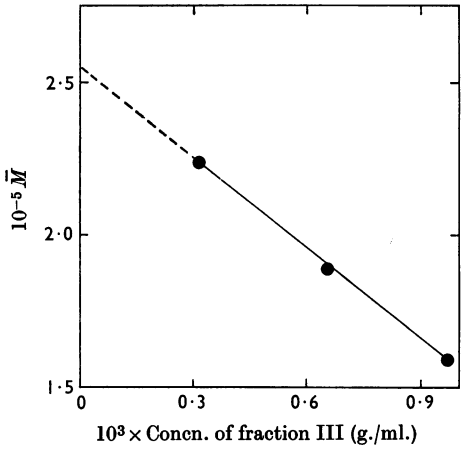

Fig. 4. Concentration dependence of the average molecular weight of fraction III. Average molecular weights (M) were calculated from the slopes of the plots of the logarithm of fringe displacement (μ) against R^2 (Fig. 3).

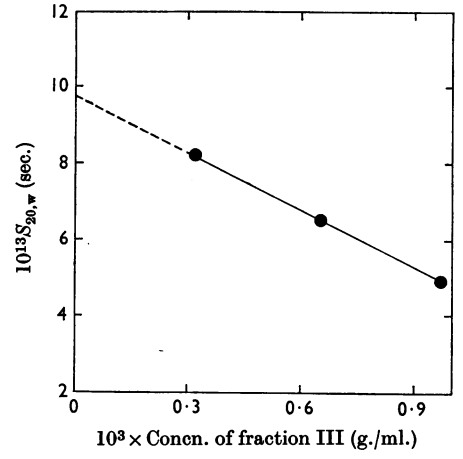

Fig. 5. Variation of the sedimentation coefficients of fraction III with concentration.

upwards. It seems likely that in the present case these two effects approximately cancel. The average molecular weight, obtained by extrapolation to zero concentration of the data given in Fig. 4, is 250000.

The sedimentation coefficient shows considerable concentration-dependence (Fig. 5), and the spreading of this boundary at low concentrations indicates that the material is in fact considerably polydisperse. The concentration-dependence of sedimentation coefficient indicates that the molecule is non-compact in form, and this would cause it to have appreciable thermodynamic non-ideality.

Specific refractive increment of fraction III. The value for this (0·162ml./g.) is consistent with the material being a chondroitin sulphate–protein complex, but is somewhat lower than the values reported by Mathews & Lozaityte (1958) for other chondroitin sulphate–protein complexes.

Other fractions. Although characterization of these is still incomplete, it seems clear that fraction II consists of hyaluronic acid, containing not more than 2% of protein. The form of its schlieren pattern in equilibrium sedimentation in a density gradient (Fig. 1*b*) is that to be expected of material which is thermodynamically highly non-ideal. It seems likely that fraction I contains most of the protein of UFR; it appears also to contain some uronic acid (Fig. 2); but, because of incomplete recovery from the tubes (see above), we do not yet know its exact composition.

GENERAL DISCUSSION

It is, perhaps, not surprising to find a proportion of chondroitin sulphate in normal synovial fluid, since the fluid is in close contact with articular cartilage, which contains large amounts of chondroitin sulphates. The fact that the mean molecular weight of our material (about 250000) is less than those of the largest complexes that have been recovered from cartilage is consistent with the view that the material found in synovial fluid has originated from the cartilage. One would expect material of lower molecular weight (whether originally of lower degree of polymerization or having undergone some degradation) to escape most easily from the cartilage. Its presence in synovial fluid serves no obvious physiological function and could therefore be regarded as accidental. It does, however, raise the question whether the hyaluronic acid in synovial fluid arises (as is commonly assumed) entirely from the synovial membrane. D. A. Lowther (personal communication) has found that hyaluronic acid forms a substantial proportion of the polysaccharide in rabbit articular cartilage; if chondroitin sulphate can escape from cartilage into synovial fluid it might be expected that hyaluronic acid may also be able to do so.

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