Rooster comb hyaluronate–protein, a non-covalently linked complex*

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Hyaluronate from rooster comb was isolated by ion-exchange chromatography on DEAE-cellulose from tissue extracts and papain digests. The preparations were labelled with [¹⁴C]acetic anhydride and subjected to CsCl-density-gradient centrifugation in 4 M-guanidinium chloride in the presence and absence of 4% ZwittergentTM 3-12. A radioactive protein fraction was separated from the hyaluronate when the zwitterionic detergent was also present. The protein could also be separated from the glycosaminoglycan by chromatography on Sepharose CL-6B eluted with the same solvent mixture. The protein fraction contained three protein bands of M_r 15000–17000 as assessed by polyacrylamide-gel electrophoresis in 0.1% SDS, and seemed to lack lysozyme activity. No evidence of other protein or amino acid(s) covalently linked with the hyaluronate was obtained. The hyaluronate–protein complex may be re-formed upon mixing the components, the extent of its formation depending on the conditions used. The results show that, as in chondrosarcoma [Mason, d'Arville, Kimura & Hascall (1982) *Biochem. J.* **207**, 445–457] and teratocarcinoma cells [Prehm (1983) *Biochem. J.* **211**, 191–198] the rooster comb hyaluronate also is not linked covalently to a core protein.

INTRODUCTION

Highly purified preparations of hyaluronate from various sources contain small amounts of protein, ranging from 0.2 to 3% by weight. The protein is firmly associated with the polysaccharide and cannot be separated from it by 4 M-guanidinium chloride or other dissociative procedures (Sandson & Hammerman, 1962; Swann, 1968b; Laurent, 1970; Greiling *et al.*, 1970; Scher & Hammerman, 1972; Hardingham & Muir, 1974; Cleland & Sherblom, 1977; Mikuni-Takagaki & Toole, 1981; Kalpaxis *et al.*, 1985). On the basis of such evidence, Mikuni-Takagaki & Toole (1981) have proposed that Rous-sarcoma-virus-transformed chick-embryo fibroblasts synthesize a covalently linked hyaluronate–protein complex. The protein has a M_r of about 12000 and can be adsorbed on Sephadex G-25 after digestion of the glycosaminoglycan with chondroitinase ABC. On the other hand, data from two different laboratories indicate that the synthesis of hyaluronate by primary cultures of chondrocytes from Swarm rat chondrosarcoma (Mason *et al.*, 1982) or by differentiated tetracarcinoma cells (Prehm, 1983) proceeds without the involvement of a protein. Furthermore, it has been shown (Mason *et al.*, 1982) that small amounts of protein/peptide associated with extracellular hyaluronate (cultured medium) can be removed from it by centrifugation in 4 M-guanidinium chloride/4% ZwittergentTM 3-12. It therefore appears that the association of hyaluronate with protein(s) becomes relative to the 'dissociative' procedures employed.

We have utilized the protein/peptide component of hyaluronate isolated from papain digests of rooster comb

to immobilize the polysaccharide on cellulose fibres (Kalpaxis *et al.*, 1985); the hyaluronate remains bound on the fibres even when 4 M-guanidinium chloride is used, indicative of a strong association. However, in view of the strong dissociative properties of ZwittergentTM 3-12/guanidinium chloride (Kimura *et al.*, 1981; Mason *et al.*, 1982), we thought it necessary to re-examine the status of rooster comb hyaluronate.

In the present paper we report data indicating that extracellular hyaluronate from rooster comb is non-covalently associated with three proteins of closely similar electrophoretic mobility (M_r 15000–17000), which can be separated from it only by the combined action of ZwittergentTM 3-12 and guanidinium chloride. The association appears to be reversible and the protein moiety appears to lack lysozyme activity.

MATERIALS AND METHODS

Materials

Sepharose CL-6B, Sephadex G-25, Dowex 50X8 (H⁺ form; 200–400 mesh), Fluorinert FC-40, DEAE-cellulose (microgranular form, capacity 0.87 mequiv./g), guanidinium chloride, CsCl, *Micrococcus lysodeikticus* proteinase K and papain (twice recrystallized) were purchased from Sigma Chemical Co. Chondroitinase ABC was from Miles Laboratories, ZwittergentTM 3-12 from Calbiochem–Behring Corp., [¹⁴C]acetic anhydride [(20% (w/w) in toluene; sp. radioactivity 19.7 Ci/mol] from Amersham International, and egg-white lysozyme (three times recrystallized) from BDH.

* Dedicated to Helen Muir on her 65th birthday.

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Methods

Uronic acid was determined by the borate-carbazole reaction (Bitter & Muir, 1962), protein by Folin reaction (Lowry *et al.*, 1951), and *N*-acetylglucosamine by a modification (Reissig *et al.*, 1955) of the Morgan-Elson reaction. Amino acid and hexosamine analyses were performed in a Beckman model 120C amino acid analyser after hydrolysis of the samples in 6 M-HCl at 110 °C for 20 h (Tsiganos & Muir, 1969) or in 8 M-HCl at 95 °C for 3 h (Swann & Balazs, 1966) under N₂, respectively. Because of large amounts of glucosamine in the samples used for amino acid analysis, which interfere with the analysis of lysine and histidine, the hydrolysates were first passed through a Dowex 50X8 (H⁺ form) column eluted with 0.3 M- and 1 M-HCl. The acidic and neutral amino acids and glucosamine were recovered in the first 5 column vol. of 0.3 M-HCl, and the basic amino acids were eluted with 1 M-HCl. Each fraction was dried and analysed separately.

Isolation of hyaluronate

Hyaluronate from rooster combs (White Leghorn) was prepared by two procedures. The tissue from about 2-months-old roosters was processed within 6 h after death, being kept in ice all the time until used.

Procedure I. The tissue was extracted essentially as described by Swann (1968a) and the hyaluronate was isolated by ion-exchange chromatography. Briefly, fresh tissue was passed through a mincing machine and then extracted for 10 days with 10 vol. of 0.05 M-sodium acetate, pH 5.8, at 4 °C under toluene. The extraction solution contained the proteinase inhibitors 6-amino-hexanoic acid (0.1 M), benzamidinium hydrochloride (5 mM) and disodium EDTA (10 mM), and was replaced daily. The pooled extracts were concentrated, freed of proteins and any nucleic acids by successive precipitation with 10% (w/v) trichloroacetic acid and 90%-satd (NH₄)₂SO₄ respectively (Cássaro & Dietrich, 1977) and then subjected to ion-exchange chromatography on DEAE-cellulose eluted with a gradient of sodium acetate, pH 5.8 (0.1–2 M), in 6 M-urea (Kapoor *et al.*, 1981). The hyaluronate was recovered from the 0.55 M-sodium acetate fractions by precipitation with 5 vol. of ethanol, and is referred to below as 'HA-E'.

Procedure II. In this case, acetone-dehydrated tissue was first digested with papain, and the hyaluronate was isolated exactly as described above. This preparation is referred to below as 'HA-P'.

[¹⁴C]Acetylation of hyaluronate and proteoglycans

Samples of both preparations of hyaluronate were *N*-acetylated with [¹⁴C]acetic anhydride as described by Riordan & Vallee (1972). The samples were dissolved (20 mg/ml) in 50%-satd. sodium acetate at 0 °C, and [¹⁴C]acetic anhydride (a total of 250 µCi) was added in several lots over a period of 1½ h. To remove any unincorporated radioactivity, the hyaluronate was repeatedly precipitated with 5 vol. of cold ethanol and finally was passed through a Sephadex G-25 column eluted with 0.5 M-sodium acetate. The preparations HA-E and HA-P recovered from the void-volume fractions had specific radioactivities of 14 and 16 d.p.m./µg of uronic acid respectively. A sample of proteoglycan aggregates

prepared from chick sternal cartilage as described by Sajdera & Hascall (1969) was also [¹⁴C]acetylated in an exactly similar manner. This preparation had a specific radioactivity of 55 d.p.m./µg of uronic acid.

Equilibrium density-gradient centrifugation

Samples of the ¹⁴C-labelled hyaluronate preparations were dissolved (0.5 mg of uronic acid/ml) in 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8; enough CsCl was added to bring the density of the solution to 1.43 g/ml, and then the mixture was centrifuged in a SW 50.1 rotor (L2 65B centrifuge) for 96 h at 10 °C (Mason *et al.*, 1982). In another experiment the samples were first dissolved in 8% (w/v) ZwittergentTM 3-12, left for 2 h at 4 °C and then mixed with an equal volume of 8 M-guanidinium chloride/0.1 M-sodium acetate, pH 5.8; the density was adjusted with CsCl to 1.43 g/ml and the mixture centrifuged as above. The tubes from all centrifugations were emptied by pumping upwards Fluorinert FC-40 at a rate of 0.5 ml/min. Fractions (320 µl) were collected with the use of a drop counter, and their density, uronic acid content and radioactivity were measured.

Enzymic digestions

Chondroitinase ABC digestion was performed as described by Yamagata *et al.* (1968). Samples of hyaluronate or proteoglycans were dissolved (3 mg/ml) in 0.05 M-Tris/acetate, pH 7.3, containing 0.1 unit of chondroitinase ABC/ml and the proteinase inhibitors and incubated at 37 °C overnight.

Papain digestion was performed in 0.1 M-sodium phosphate buffer, pH 6.5, at 60 °C essentially as described by Scott (1960).

Gel electrophoresis

Polyacrylamide-gel electrophoresis in 0.1% SDS was performed as described by Laemmli (1970). The running and the stacking gels were 10% and 4% acrylamide respectively. The gels were stained with Coomassie Brilliant Blue R-250 (BDH) (0.25%, w/v) in methanol/acetic acid/water (43:7:50, by vol.) (Aletas & Tsiganos, 1985) without prior fixation in trichloroacetic acid, destained by gentle agitation in methanol/acetic acid/water (40:7:53, by vol.), and the pattern of the bands was drawn. When radioactive samples were electrophoresed, after staining and drawing the gels were frozen, cut into 2 mm slices with a multiple slicer, each slice was dissolved in 0.2 ml of 3% (v/v) H₂O₂ at 37 °C overnight, and then its radioactivity was measured.

Gel chromatography

Gel chromatography on Sephadex G-25 or Sepharose CL-6B was carried out at room temperature or at 4 °C with columns of 140 cm × 1.4 cm and 140 cm × 0.8 cm respectively. The Sephadex column was eluted with 0.02 M-ammonium acetate or 1% SDS/0.02 M-ammonium acetate (Mikuni-Takagaki & Toole, 1981), and the fractions (2.5 ml) were analysed for uronic acid and radioactivity. The Sepharose column was eluted with 0.5 M-sodium acetate, pH 7.0, or with 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, with or without 4% ZwittergentTM 3-12. Fractions (1.4 ml) were collected and analysed for uronic acid and radioactivity. The void volume (*V*₀) and total volume (*V*_t) of the columns were

determined by passing through a sample of proteoglycans and by measuring the content of the empty tube respectively. This method gives a somewhat higher V_t , particularly for Sephadex G-25, than the chromatography of $^3\text{H}_2\text{O}$.

Lysozyme activity

Samples of the protein fraction HD (Fig. 1b), equivalent to amounts derived from the centrifugation of 3.6 mg of uronic acid of HA-E, were tested for lysozyme activity as described by Jollès (1962), with a suspension of *Micrococcus lysodeikticus* as substrate. Standard solutions containing 5–26 units of egg-white lysozyme (0.1–0.5 μg of protein) were also assayed.

Reaggregation experiments

Protein-free hyaluronate was prepared by chromatography of unlabelled HA-E on Sepharose CL-6B eluted with 4 M-guanidinium chloride/4% ZwittergentTM 3-12. The hyaluronate was recovered from the void-volume fractions (results not shown) and tested for its ability to combine with the protein fraction HD (Fig. 1b). Hyaluronate (1 mg of uronic acid) in 0.5 M-sodium acetate, pH 7.0, was mixed with an amount (3600 d.p.m.) of [^{14}C]HD equivalent to that derived from the centrifugation of [^{14}C]HA-E (1 mg of uronic acid), left at 4 °C overnight with mild shaking and then chromatographed on Sepharose CL-6B eluted with 0.5 M-sodium acetate, pH 7.0. After 1 column vol. of this buffer had passed through, the column was eluted with 4 M-guanidinium chloride/4% ZwittergentTM 3-12. All fractions were monitored for uronic acid and radioactivity. Those containing the uronic acid (Fig. 4a) were pooled, concentrated, measured for radioactivity, made to 4 M-guanidinium chloride and then chromatographed on the same column equilibrated and eluted with 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8. The uronic acid-containing fractions from this chromatography were also pooled, analysed as above, made to 4% ZwittergentTM 3-12 and applied to the same column eluted with 4 M-guanidinium chloride/4% ZwittergentTM 3-12, and all fractions were analysed as above.

RESULTS AND DISCUSSION

Repeated extraction of rooster combs with dilute buffer for 10 days brought into solution about 75% of its total uronic acid content, an amount comparable with that extracted by Swann (1968a) by a similar procedure. The pooled extracts and the digest were treated with 10% trichloroacetic acid and 90% -satd. $(\text{NH}_4)_2\text{SO}_4$, which precipitated most extraneous proteins and nucleic acids respectively as assessed by absorption measurements at 280 and 260 nm. Losses of about 10% of the uronic acid were also encountered during this procedure. After precipitation with trichloroacetic acid and $(\text{NH}_4)_2\text{SO}_4$ the extract and digest were dialysed exhaustively against water, concentrated, made to 6 M-urea and chromatographed on DEAE-cellulose eluted with a gradient of increasing ionic strength in 6 M-urea (results not shown). The hyaluronate, which was recovered from the 0.55 M-sodium acetate fractions, appeared to be of high purity, since the only hexosamine it contained was glucosamine, in almost equimolar amounts with uronic acid. The size of the amino acid analyser column and that

Fig. 1. CsCl-density-gradient centrifugation of hyaluronate

(a) [^{14}C]Acetylated rooster comb HA-E hyaluronate preparation was centrifuged in 4 M-guanidinium chloride/50 mM-sodium acetate, pH 5.8, at starting density 1.43 g/ml; (b) fractions 7–16 (ρ 1.38–1.6 g/ml) from (a) were pooled, made to 4% Zwittergent and centrifuged as before; (c) fractions 7–16 from (b) were pooled and centrifuged as in (b). For further details see the text. —, [^{14}C]Acetate radioactivity; ----, uronic acid; ●—●, density.

of the sample used for hexosamine analysis would have detected at least 1 residue of galactosamine in 100 residues of glucosamine.

Each preparation of the hyaluronate contained small amounts of protein, determined by the Lowry *et al.* (1951) method, 0.25 and 0.20% (w/w) for HA-E and HA-P respectively. These values, however, may be quite inaccurate, since a mixture of 5 mg each of *N*-acetylglucosamine and glucuronolactone or 12 mg of Zwittergent-purified hyaluronate, when analysed with the same method, produced colour corresponding to 0.15% (w/w) protein content.

Association of protein with hyaluronate

Each preparation of ^{14}C -labelled hyaluronate was centrifuged in a linear density gradient of CsCl in 4 M-guanidinium chloride. Under these conditions, which have been referred to as dissociative for proteoglycan-hyaluronate complexes, all radioactivity and uronic acid were distributed as a single band in the density range 1.4–1.6 g/ml (Fig. 1a). Assuming that most of the acetic

anhydride had reacted with the protein/peptide moiety, these results confirm previous reports (Scher & Hammerman, 1972; Cleland & Sherblom, 1977; Mikuni-Takagaki & Toole, 1981) on the strong association of the protein with the hyaluronate. However, when fractions 7–16 from [14 C]HA-E (Fig. 1a) were pooled, made also to 4% ZwittergentTM 3-12 and starting density 1.43 g/ml and centrifuged as above, a distinct radioactive band at the top of the gradient was separated from the glycosaminoglycan (Fig. 1b), which was found in the same fractions as before. The low-density fraction, which will be referred to as HD, contained 28% of the applied radioactivity (3600 d.p.m. from 1 mg of uronic acid), the rest being co-sedimented with the hyaluronate. No further radioactivity was separated from the uronic acid-containing material (Fig. 1c) when fractions 7–16 (Fig. 1b) were pooled and centrifuged again in 4 M-guanidinium chloride/4% ZwittergentTM 3-12, indicating that, whatever molecule contained the label, either it was intimately associated with the hyaluronate or it had exactly the same buoyant density as the glycosaminoglycan. This aspect was investigated further (see below).

A distribution of radioactivity and uronic acid similar to that for [14 C]HA-E was also obtained when preparation [14 C]HA-P was centrifuged in 4 M-guanidinium chloride/4% ZwittergentTM 3-12, except that the low-density fraction contained 4% of the applied radioactivity (results not shown). Again, no further radioactivity was separated from the hyaluronate on re-centrifugation as above. The hyaluronate recovered from the centrifugation in guanidine/Zwittergent will be referred to as 'purified hyaluronate'.

The separation of the protein from hyaluronate was also studied by chromatography. Samples of [14 C]HA-E in 4 M-guanidinium chloride/4% ZwittergentTM 3-12 were applied on a Sepharose CL-6B column eluted with the same solvent mixture. A profile of uronic acid and radioactivity similar to that shown in Fig. 4(c) was obtained. About 30% of the radioactivity was eluted with the total-volume fractions, whereas the rest co-chromatographed with the hyaluronate, in agreement with the data obtained from centrifugation in guanidine/Zwittergent.

It has been reported by Mikuni-Takagaki & Toole (1981) that the protein associated with hyaluronate from the culture medium of transformed fibroblasts could be adsorbed on Sephadex G-25 after degradation of the glycosaminoglycan with chondroitinase ABC and that it was only eluted from the gel with buffer containing 1% SDS. To find out if the protein in rooster comb hyaluronate behaves in the same way, samples of [14 C]HA-E and [14 C]HA-P were first digested with chondroitinase ABC and then applied to a Sephadex G-25 column. The column was first eluted with 1 bed vol. of 0.02 M-ammonium acetate and then with the same buffer containing 1% SDS. About 30% and 5% of the radioactivity in [14 C]HA-E and [14 C]HA-P were eluted with the front of 1% SDS respectively, which are about the same as the proportions separated in each case by centrifugation (Figs. 2a and 2b). It appears that the proteins in rooster comb and in transformed-fibroblast-culture-medium hyaluronate have at least this property in common, which might govern their interaction with the glycosaminoglycan.

In contrast, no radioactivity was retained by the gel when a digest of 15 mg of purified hyaluronate from [14 C]HA-E was also chromatographed under the same

Fig. 2. Gel chromatography on Sephadex G-25 of chondroitinase ABC digests of hyaluronate preparations

Experimental details are given in the text. (a) Digest of 2.5 mg of [14 C]HA-E; (b) digest of 2.5 mg of [14 C]HA-P; (c) digest of 2.8 mg of hyaluronate isolated by centrifugation of [14 C]HA-E in guanidine/Zwittergent (Fig. 1b). The columns were first eluted with 0.02 M-ammonium acetate and then with the same buffer containing 1% SDS, as indicated by arrows. The inserts show drawings of protein bands obtained after electrophoresis, on 10%-polyacrylamide gels in 0.1% SDS, of pooled fractions as indicated by the bars. —, [14 C]Acetate radioactivity; ----, uronic acid; V_0 , void volume; V_t , total volume.

conditions (Fig. 2c), confirming the results obtained upon re-centrifugation of the hyaluronate in guanidine/Zwittergent.

On electrophoresis of the 1% SDS eluates on 10%-polyacrylamide gels in 0.1% SDS, three bands of the same intensity were detected in each sample (Figs. 2a and 2b). The mobilities of these protein bands, as well as their radioactivity patterns obtained after cutting and measuring the gels, were the same as those of the bands detected in the centrifugation fraction HD (see below). These results indicate that any of the above procedures, i.e. centrifugation, or chromatography on Sepharose CL-6B in 4 M-guanidinium chloride/4% Zwittergent, or chromatography of chondroitinase digest on Sephadex G-25 in 1% SDS, may be used to isolate the protein that is non-covalently linked with hyaluronate.

Absence of covalently linked protein

The radioactivity in all chondroitinase digests which did not bind on Sephadex G-25 co-chromatographed with the main degradation product, the disaccharides (Fig. 2). The structure of this fraction was determined by *N*-acetylglucosamine and Δ -4,5-unsaturated uronic acid analyses, the latter being carried out by the method of Adams & Muir (1980). No indication of a separate radioactive peak was observed, suggesting the absence or near absence of any protein/peptide in the samples other than that found in the 1% -SDS eluates. However, in view of the results obtained with fraction HD, the possibility of the presence of an unlabelled protein could not be excluded, and this was investigated by subjecting the void-volume fractions (Fig. 2) from each chromatography to 10% -polyacrylamide-gel electrophoresis in 0.1% SDS. The only protein detected in these fractions (results not shown) was albumin, which is an additive to the chondroitinase preparations, suggesting the absence of any protein large enough to be excluded by the gel.

In order to find out whether any of the radioactivity in the disaccharides was due to amino acid(s), a large amount of disaccharides was subjected to amino acid analysis. A sample of disaccharides (9 mg of uronic acid) derived from the digestion of purified hyaluronate from [14 C]HA-E was hydrolysed in 45 ml of 6 M-HCl at 110 °C for 20 h under N_2 , and the dried hydrolysate passed through a Dowex 50 $\times$ 8 (H^+ form) column eluted in succession with 5 and 3 column vol. of 0.3 M- and 1 M-HCl respectively. No amino acid was detected in either of the eluates when they were analysed for amino acids. The Dowex-column procedure would have facilitated the determination of basic amino acids, since all glucosamine

in the hydrolysate was eluted with 0.3 M-HCl and affected only the analysis of phenylalanine. If all the radioactivity in the disaccharides represented *N*-acetylated amino acids, then, from the specific radioactivity of the acetic anhydride used, the sample analysed would contain about 2 μ mol of amino acids, an amount measurable in the amino acid analyser. On the other hand, if there were only one amino acid residue in each hyaluronate molecule, then, assuming that rooster comb hyaluronate has M_r 1.2×10^6 (Swann, 1968a), 9 mg of uronic acid disaccharides would contain about 17 nmol of amino acid, which also can be detected. By using large volumes of 6 M-HCl for hydrolysis, the extent of destruction of amino acids is expected to have been decreased, thus making the analytical data more reliable.

The failure to detect by electrophoresis any protein after chondroitinase digestion or any amino acid in the disaccharide fraction, which represents about 98% of the hyaluronate, suggests that the only protein present in the hyaluronate is the non-covalently linked moiety.

Since no amino acids were detected in the disaccharides, it appears that, under the acetylation conditions used, the glycosaminoglycan is most probably acetylated on the hydroxy residues. This is not confined to hyaluronate alone, since disaccharides from [14 C]acetylated proteoglycans or from their chondroitin sulphate chains isolated by alkali treatment and chromatography on DEAE-cellulose contained radioactivity and in amounts/uronic acid similar to those found in the fractions from hyaluronate.

Characterization of fraction HD

Samples of the low-density fraction HD separated from [14 C]HA-E were dialysed against large volumes of Tris buffer, alone or containing 2% SDS, in dialysis tubing previously heated at 95 °C for 48 h to decrease its porosity (Callanan *et al.*, 1957). The dialysis residue was then concentrated by ultrafiltration through an Amicon YM-2 membrane, and then electrophoresed on 10% -polyacrylamide gels in the presence of 0.1% SDS. Three distinct protein bands of the same intensity and of mobilities corresponding to M_r 15000–17000 were obtained, the fastest being slightly slower than that of egg-white lysozyme (Fig. 3). When the gels were cut into slices and their radioactivity was measured, almost all of the applied radioactivity was found in those corresponding to the two fastest bands, the slowest containing little or none (Fig. 3). Since acetylation was not performed under strong denaturing conditions, it is likely that the larger protein may be particularly hidden in the molecular domains of hyaluronate, thus becoming inaccessible to the labelling reagent. The possibility that this protein might lack completely any free amino groups seems rather remote. From the intensity of the electrophoresis bands it was assessed that the amount of protein separated from HA-E was of the order of 1.5 μ g/mg of hyaluronate. If this value is true, and assuming that rooster comb hyaluronate has M_r 1.2×10^6 (Swann, 1968a), then the molar ratio of hyaluronate to protein would be about 10:1.

The protein nature of fraction HD was further established by incubating a sample with proteinase K (2 μ g of enzyme/2000 d.p.m.) as described by Mikuni-Takagaki & Toole (1981) and then chromatographing it on Sephadex G-25 eluted with 0.5 M-sodium acetate, pH 7.0. An included peak near the total volume of the

Fig. 3. SDS/polyacrylamide-gel electrophoresis of protein from hyaluronate

Experimental details are given in the text. At the top is shown a drawing of the protein bands obtained when fraction HD (Fig. 1b) was electrophoresed on 10% polyacrylamide gels in 0.1% SDS. The arrows indicate the position of proteins of known M_r when electrophoresed in the same way: (a) cytochrome *c* (12400), (b) egg-white lysozyme (14300), (c) myoglobin (17800), (d) chymotrypsinogen (25000). At the bottom is shown the distribution along the gel (top) of [14 C]acetate radioactivity determined after slicing and measuring the slices.

column (results not shown), containing all the applied radioactivity, was obtained.

When a sample (4000 d.p.m.) of [^{14}C]HD in 0.2 M-ammonium acetate was passed through a 5 ml Sephadex G-25 column eluted in succession with 1 column vol. each of acetate buffer and acetate/1% SDS, all radioactivity was found in the second eluate, which is consistent with the results obtained with chondroitinase digests. The same phenomenon was observed when HD in 0.5 M-sodium acetate, pH 7.0, was passed through Sepharose CL-6B; in this case it was eluted from the gel with 4 M-guanidinium chloride/4% ZwittergentTM 3-12 (results not shown).

It has been reported (K. E. Kuettner, unpublished work cited by Mason *et al.*, 1982) that hyaluronate from various sources contains lysozyme, which is gradually removed from it by repeated dissociative centrifugation. When a sample of fraction HD (about 3 μg of protein), freed of Zwittergent by repeated ultrafiltration through an Amicon YM-2 membrane, was mixed and incubated with *Micrococcus lysodeikticus*, no change in the absorbance of the suspension was observed over a period of 2 h. Standard mixtures containing 0.1–0.5 μg of egg-white lysozyme produced a fall in the A_{650} of 0.05–0.25 unit in the first 10 min. Control experiments with lysozyme in 4 M-guanidinium chloride/4% Zwittergent/CsCl kept at 10 °C for 96 h and then treated exactly as for fraction HD showed that under these conditions the enzyme retains all its activity. These observations, together with the absence of any radioactive material on the top of the gradient when [^{14}C]HA-E was centrifuged in 4 M-guanidinium chloride (Fig. 1a), and the electrophoretic pattern of fraction HD lead to the suggestion that the protein isolated from rooster comb hyaluronate may not be lysozyme. Further confirmation may be necessary with the use of more sensitive techniques.

Recombination experiments

The ability of the protein to recombine with hyaluronate once it was removed from it and its specificity toward this glycosaminoglycan was studied. A sample of ^{14}C -labelled HD was mixed with an equivalent amount of unlabelled purified hyaluronate in 0.5 M-sodium acetate, pH 7.0, left at 4 °C overnight with gentle shaking and then chromatographed on Sepharose CL-6B eluted with the same buffer. About 20% of the radioactivity was eluted with the hyaluronate (Fig. 4a), the rest being retained by the gel, from which it was recovered when 1 column vol. of 4 M-guanidinium chloride/4% ZwittergentTM 3-12 was passed through. The bound protein could not be removed from the hyaluronate when the void-volume fractions from the previous chromatography were pooled and chromatographed on the same column, eluted this time with 4 M-guanidinium chloride/50 mM-sodium acetate, pH 5.8 (Fig. 4b). However, when the same sample was chromatographed in 4 M-guanidinium chloride/4% ZwittergentTM 3-12, all radioactivity was found in the total-volume fractions, indicating that the complex formed was quantitatively similar to that found in the tissue.

The extent of complex-formation was improved when twice the equivalent amount of HD was mixed with hyaluronate in 4 M-guanidinium chloride/4% ZwittergentTM 3-12 and dialysed in succession against 10 vol. each

Fig. 4. Gel chromatography on Sepharose CL-6B of hyaluronate recombined with its protein

Experimental details are given in the text. (a) Purified hyaluronate in 0.5 M-sodium acetate, pH 7.0, mixed with an equivalent amount of ^{14}C -labelled fraction HD. The column was eluted first with the same buffer and then with 4 M-guanidinium chloride/4% Zwittergent, as indicated by the arrow; (b) the uronic acid-containing fractions of (a) on the same column eluted with 4 M-guanidinium chloride; (c) the same as (b), except that the eluent contained also 4% Zwittergent. —, [^{14}C]Acetate radioactivity; ----, uronic acid; V_0 , void volume; V_t , total volume.

of 2 M- and 0.5 M-guanidinium chloride/50 mM-sodium acetate, pH 5.8. About 60% of the radioactivity now co-chromatographed with hyaluronate (results not shown) when the mixture was chromatographed on Sepharose CL-6B eluted with 0.5 M-sodium acetate, pH 7.0. It should be pointed out that, because the dialysis was performed against limited volumes of solvent, there was always some Zwittergent in the environment, which might have facilitated the formation of the complex. In contrast, when the above mixture was dialysed against large volumes of 0.5 M-sodium acetate, pH 7.0, only 20% of the complex was formed. These observations suggest that complex-formation depends not so much on concentrations but more so on the existence of an environment, perhaps hydrophobic, favourable for the proteins to react with the glycosaminoglycan and to avert

possible self-association. This aspect needs to be studied further.

The interaction of the protein fraction with hyaluronate appears to be specific to this glycosaminoglycan, and seems not to be due to mere electrostatic forces. When it was mixed with chondroitin sulphate chains in 4 M-guanidinium chloride/4% ZwittergentTM 3-12, dialysed against 2 M- and 0.5 M-guanidinium chloride/50 mM-sodium acetate, pH 5.8, and chromatographed as above, no radioactivity was co-eluted with the chondroitin sulphate (results not shown).

General discussion

The results outlined in this study correlate with the evidence that hyaluronate in cultured chondrocytes from chondrosarcoma (Mason *et al.*, 1982) and in differentiated teratocarcinoma cells (Prehm, 1983) is not synthesized on a protein core. The only protein that could be detected in the hyaluronate from rooster comb was a non-covalently linked moiety separable only by the action of guanidine/Zwittergent. This protein appears to be protected to some extent by the glycosaminoglycan, since it was found in hyaluronate isolated after digestion of the tissue with papain.

The properties of the hyaluronate-protein complex, namely stability in 4 M-guanidinium chloride, dissociation in guanidine/Zwittergent, and the binding of the protein on Sepharose and Sephadex, imply that some kind of hydrophobic interactions may govern the formation and stability of the complex.

The similarity of the rooster comb hyaluronate protein to that of hyaluronate from transformed chick-embryo fibroblasts (Mikuni-Takagaki & Toole, 1981) and a protein/peptide from chondrosarcoma cells, i.e. adsorption on Sephadex and dissociation with guanidine/Zwittergent respectively, suggests that it may be present also in hyaluronate from other sources, bound to it through a common mechanism, and involved in some general biological phenomenon.

The protein may be associated with hyaluronate post-synthetically either on its way out from the cell or extracellularly. Its potential to bind through hydrophobic interactions would rather confine it on the membranous structures of the cell, thereby interacting with the glycosaminoglycan before it is shed out of the cell.

The actual role of the protein and its effect on the overall structure of the hyaluronate is not yet clear and needs be elucidated. From preliminary results from chromatography of hyaluronate on Sepharose 2B, it

appears that its removal does not affect the hydrodynamic volume of the glycosaminoglycan.

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