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Extraction and purification of proteoglycans from mature bovine bone

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Proteoglycans were extracted in good yields from the mineralized matrix of ground bovine bone, by using a two-step extraction procedure. Proteoglycans (8% of total), not associated with the bone mineral, were extracted at −20°C with 4M-guanidinium chloride containing proteinase inhibitors. Proteoglycans associated with the mineral, which accounted for 60% of the total, were then solubilized when EDTA was added to the extraction solvent. They were fractionated and purified in the presence of 4M-guanidinium chloride by CsCl-density-gradient centrifugations followed by chromatography on Sepharose CL-4B. Further purification was obtained by chromatography on DEAE-cellulose and hydroxyapatite in the presence of 7M-urea. Three populations of proteoglycans and additional glycosaminoglycan peptides were obtained. The molecular dimensions of both intact molecules and of their side chains as well as their amino acid composition were different, indicating that they represent separate molecular entities. The main proteoglycan self-aggregated in the absence of 4M-guanidinium chloride or 7M-urea, a property that was abolished when the proteoglycan core protein was fragmented.

Bone resists various mechanical forces, because of an ideal structural combination of its inorganic and organic constituents. In the development and growth of long bones, osteoblasts lay down unmineralized bone tissue (osteoid) on remnants of calcified cartilage, a process known as endochondral ossification (for review see Bloom & Fawcett, 1975). The osteoid is mineralized after some 10 days, i.e. about 70% of its organic matrix is replaced by calcium phosphate, mainly hydroxyapatite crystals. The mechanical properties of bone are largely determined by the size, shape and orientation of the hydroxyapatite crystals, which in turn depend on the molecular architecture of the collagen fibrils (Glimcher, 1981). More than 90% of the remaining organic matrix is type I collagen (Skinner, 1979), but the matrix also contains a number of highly polyanionic molecules such as phosphoproteins (Shuttleworth & Veis, 1972; Spector & Glimcher, 1972), sialoprotein (Andrews *et al.*, 1967) and proteoglycans (Herring, 1968; Engfeldt & Hjerpe, 1976; Reddi *et al.*, 1978). These molecules may also have effects on the formation of the hydroxyapatite crystals.

Proteoglycans have been identified in extracts of bone (Herring, 1968; Engfeldt & Hjerpe, 1976; Reddi *et al.*, 1978). Little is, however, known about their chemical and structural composition, as techniques allowing isolation of intact bone-specific proteoglycans in good yield have not been available. A major problem has been to liberate the proteoglycans from their association with the bone mineral. Furthermore, bone, being vascularized, contains significant amounts of blood-vessel-wall proteoglycans. Extracts would therefore contain significant proportions of such proteoglycans. Other problems involve isolation of intact molecules, a consequence of the presence in bone of a large number of blood cells rich in proteinases, capable of degrading the proteoglycans during preparation.

The aim of the present investigation has been to design procedures for extraction and purification of intact proteoglycans from the mineralized matrix of mature bovine bone. A preliminary report of some of the data has already been presented (Franzén & Heinegård, 1983).

Materials and methods

Chemicals

Guanidinium chloride (practical grade), papain (type III, 2 × crystallized) and chondroitinase ABC were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sepharose CL-4B, Sepharose 6B and molecular-mass calibration kits were pro-

ducts of Pharmacia Fine Chemicals, Uppsala, Sweden. DE-52 anion-exchange DEAE-cellulose was bought from Whatman Chemicals, Maidstone, Kent, U.K. Hydroxyapatite for chromatography was from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

All other chemicals used were of analytical grade. Before use the guanidinium chloride solutions were passed through a column of activated charcoal to remove u.v.-absorbing material. All urea solutions were passed through an anion/cation-exchange resin and buffered immediately before use.

Tissues

Diaphyses from 2-year-old bulls were obtained immediately after slaughter and handled at temperatures of 0–4°C. Within an hour, muscles, bone marrow and other contaminating tissues had been removed and the bone had been crushed with a hammer, frozen in liquid N_2 and ground while still frozen in a Wiley mill. The frozen powder was fractionated at −20°C according to size by using metal sieves. Particles of sizes from 60 to 120 mesh were recovered and kept at −60°C until extraction. Analytical data were, however, similar for particles of larger size, although extraction yields were somewhat lower.

Isolation of proteoglycan

Sequential extraction. In a typical experiment, 50g (wet wt.) of frozen bone powder (60–120 mesh) was extracted at −20°C for 6h with 10vol. (500ml) of precooled extraction medium I, i.e. 4M-guanidinium chloride/50mM-sodium acetate buffer, pH5.8, containing the proteinase inhibitors 0.1M-6-aminohexanoic acid, 5mM-benzamidinium chloride (Oegema *et al.*, 1975) and 5mM-*N*-ethylmaleimide, the last mainly to prevent disulphide exchange (Heinegård *et al.*, 1981). Extract and residue were rapidly separated by vacuum filtration through a nylon sieve, and the residue was washed three times with 50ml of ice-cold extraction medium.

The residue was next extracted at 4°C for 24h with 60vol. (3000ml) of extraction medium II, i.e. 4M-guanidinium chloride/50mM-Tris/HCl buffer, pH7.4, containing 0.1M-6-aminohexanoic acid, 5mM-benzamidinium chloride, 5mM-*N*-ethylmaleimide and 0.25M-EDTA. The extract was clarified by centrifugation at 10000*g* ($r_{av.}$ 8.6cm) for 40min. The pellet was washed twice with 300ml of cold extraction medium II and again centrifuged as described above. The supernatants were pooled to form extract II.

CsCl-density-gradient centrifugation. The extract II was concentrated to about 120ml by ultrafiltration at 4°C over a PM-10 ultrafilter (Amicon Corp., Lexington, MA, U.S.A.). Solid CsCl was added to the preparation to give a density of 1.35g/ml, and the preparation was centrifuged in an MSE ultracentrifuge with an 8×25ml (20ml/tube) aluminium rotor at 35000rev./min (85000*g*, $r_{av.}$ 6.44cm) for 60h at 12°C. The tubes were emptied by gravity from the bottom into five equal 4ml fractions, designated D1 (bottom) to D5 (top).

Dissociative gel chromatography. A sample (20ml) of the bottom fraction (D1) from the CsCl-density-gradient centrifugation was dialysed for 24h at 4°C against two changes of 250ml of 4M-guanidinium chloride/50mM-sodium acetate buffer, pH5.8, to remove the CsCl. The dialysis residue was then concentrated to 2ml by ultrafiltration over an Amicon PM-10 filter and chromatographed on a Sepharose CL-4B column (1.5cm×140cm) eluted at 6ml/h with 4M-guanidinium chloride/50mM-sodium acetate buffer, pH5.8.

High-density CsCl-gradient centrifugation. The pooled fraction (4B-III) from the dissociative Sepharose CL-4B gel chromatography was concentrated by ultrafiltration over an Amicon PM10 filter, and the density of the retentate was adjusted to 1.42g/ml by adding solid CsCl.

The solution was centrifuged in a Beckman 70.1Ti angle rotor (13ml/tube; 2mg/ml) at 45000rev./min (140000*g*, $r_{av.}$ 6.1cm) for 60h at 12°C. The tubes were emptied by pumping the solution from the bottom with a peristaltic pump, and 0.5ml fractions were collected. The densities were determined as described above.

Anion-exchange chromatography. The pooled fraction (DIII, containing 20mg of material), from the second CsCl-density-gradient centrifugation was dialysed for 36h at 4°C against three changes of 100vol. of 7M-urea/10mM-Tris/HCl/0.1M-sodium acetate buffer, pH6.0. The dialysis residue was chromatographed on a DEAE-cellulose (DE-52) column (1.0cm×15cm) eluted at 4°C at 10ml/h with 7M-urea/10mM-Tris/HCl/0.1M-sodium acetate buffer, pH6.0. Material bound to the column was eluted with a linear gradient from 0.1M- to 1.2M-sodium acetate in the urea solution, pH6.0.

Hydroxyapatite chromatography. A sample (10ml, 1.5mg/ml) of the pooled fraction (DE52-III) from the DE-52 chromatography was dialysed for 24h at 4°C against two changes of 250ml of 7M-urea/10mM-Tris/HCl/10mM-sodium phosphate buffer, pH7.4. The dialysis residue was applied to a hydroxyapatite column (2cm×20cm) eluted at 14°C at 10ml/h with 7M-urea/10mM-Tris/HCl/10mM-sodium phosphate buffer, pH7.4. Material bound to the hydroxyapatite was eluted with a linear sodium phosphate gradient from 10mM to 0.5M in the urea solution, pH7.4.

Electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Equal-sized samples of the CsCl-gradient fractions (D1 to D5) and of the pooled fractions from the Sepharose CL-4B chromatogram (CL4B I–V) were dialysed against two changes of 1500ml of 10mM-sodium acetate/10mM-Tris/HCl buffer, pH8.0, for 48h at 4°C. To depolymerize the glycosaminoglycan chains, one half of each sample was incubated at 37°C for 6h with chondroitinase ABC (0.01 unit/mg dry wt. of proteoglycan). After addition of 1vol. of 4% (w/v) sodium dodecyl sulphate/10% (v/v) 2-mercaptoethanol in electrophoresis buffer, all samples were incubated for 2h at 37°C, and electrophoresed on polyacrylamide gels (T = 8%, C = 2.5%) in the discontinuous buffer system described by Neville (1971). Proteins were stained with 0.25% (w/v) Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.).

Agarose/polyacrylamide-gel electrophoresis. Measured samples of fractions were incubated for 18h at 4°C with 9vol. of ethanol. Precipitates formed were recovered by centrifugation, suspended in 2.5M-sodium acetate and re-precipitated with ethanol to remove traces of guanidinium chloride as described elsewhere (Paulsson *et al.*, 1983). Before electrophoresis, the precipitates were dried, dissolved in 1% (w/v) sodium dodecyl sulphate and incubated at 37°C for 2h. An equal volume of 0.02% Bromophenol Blue/60% (w/v) sucrose dissolved in electrophoresis buffer, pH6.0, was added, and the samples were electrophoresed on agarose/polyacrylamide slab gels originally described elsewhere (McDevitt & Muir, 1971) but modified such that the gels contained 0.05% (w/v) Triton X-100 (D. Heinegård, unpublished work). Gels were stained with 0.02% Toluidine Blue in 0.1M-acetic acid and de-stained in 3% (v/v) acetic acid.

Chemical methods

To determine extraction yields, measured samples of the two extracts and the residue were extensively dialysed against distilled water and freeze-dried. The samples were suspended in 50mM-sodium phosphate buffer, pH7.0, containing 75mM-disodium EDTA and 5mM-2-mercaptoethanol and digested for 24h at 60°C with 20μl of a suspension of crystalline papain (25mg of protein/ml) (Hjertquist & Vejlens, 1968). Glycosaminoglycans were isolated from the digests by using ion-exchange chromatography on DEAE-cellulose (Whatman DE-52) as described elsewhere (Axelsson & Heinegård, 1975). Columns were eluted with water, 20mM-HCl and 2M-HCl. The last fraction contains the glycosaminoglycans, which were hydrolysed in 4M-HCl (AristaR) for 10h at 100°C in tubes sealed under argon. Contents of glucosamine and galactosamine were determined with an automatic amino acid analyser. The extraction yield was calculated from the amount of glycosaminoglycan-bound hexosamine in the two extracts (I and II) and the residue.

To determine the distribution of glycosaminoglycans and proteoglycans in the CsCl-density-gradient fractionation of extract II, equal samples of fractions from the gradient (D1 to D5) were dialysed against 0.5M-NaCl and then extensively against distilled water and freeze-dried. One half of each fraction was digested with papain, and the glycosaminoglycans were quantified as described above.

The other half of the freeze-dried material was dissolved in 0.1M-NaOH, and the protein content in the fractions was determined by using the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Uronic acid contents of samples dissolved in 4M-guanidine or 4M-guanidine/CsCl were determined by the carbazole method of Bitter & Muir (1962).

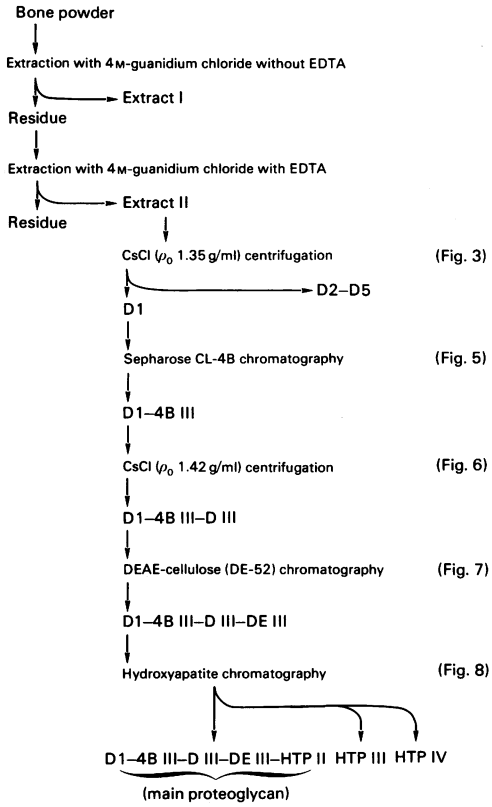
Uronic acid contents of fractions containing 7M-urea were determined by using an automated version of the carbazole method (Heinegård, 1973). Sialic acid was determined by using an automated version (Lohmander *et al.*, 1980) of the method described by Jourdain *et al.* (1971). To increase colour yields all urea solutions were diluted to 0.4M-urea with distilled water before quantification of sialic acid.

Column chromatography

An analytical Sepharose 6B column (0.3mm × 140mm) was eluted with 0.5M-sodium acetate, pH7.0, also containing bovine serum albumin (20μg/ml) as a carrier. The column was eluted at 600μl/h, and 230μl fractions were collected. Samples containing 100μg of proteoglycan were dissolved (4mg/ml) in 4M-guanidinium chloride/50mM-sodium acetate buffer, pH5.8, and then dialysed into water. One half of each sample was then freeze-dried, and the other half was mixed with double-concentration elution buffer and chromatographed on the analytical Sepharose 6B column. The freeze-dried samples were solubilized in 50mM-NaOH/1M-$NaBH_4$ (Carlson, 1968) and incubated for 48h at 45°C to liberate the chondroitin sulphate chains. Before gel chromatography on the Sepharose 6B column, the samples were neutralized with 1M-acetic acid.

Results and discussion

A flow diagram showing the various steps utilized in the purification of the proteoglycans is shown in Scheme 1.



Scheme 1. *Flow diagram showing the steps in purifying the bone proteoglycans*
For full experimental details see the Materials and methods section.

Extraction

Bone is composed of a mineralized matrix with additional components of soft connective tissue, blood vessels and bone marrow. A preparation of proteoglycans from bone would therefore contain molecules from all these tissue components. Most connective-tissue proteoglycans, however, can be extracted in good yield with 4M-guanidinium chloride (Antonopoulos *et al.*, 1974), a solvent that will not dissolve the hydroxyapatite crystals. The strategy chosen in the present study was therefore to first extract 'non-specific' proteoglycans from pulverized bone with 4M-guanidinium chloride and in a second step, by adding EDTA to the extracting solvent, dissolve the mineral containing bound proteoglycans. A similar approach was used by Fischer *et al.* (1983), who extracted subperiostal bone pieces from immature bovine animals. To prevent proteolytic fragmentation of the macromolecules a number of proteinase inhibitors were included in the extraction media, and importantly the extraction was performed at very low temperature, −20°C. This first extract (I) would contain any proteinase present, in addition to proteoglycans not associated with the calcified matrix.

The time course of the first extraction was studied. Blood-vessel-wall proteoglycans were used as markers for 'non-specific' proteoglycans, since these could be determined quantitatively by radial immunodiffusion by the method of Mancini *et al.* (1965) with antibodies directed against bovine aorta proteoglycans (S. Gardell & D. Heinegård, unpublished work). Only a 3–6h extraction was required to extract the non-specific proteoglycans (Fig. 1). At that time 8% of all the proteoglycans in the bone were extracted (Table 1). Subsequent extraction (II) with 4M-guanidinium chloride containing EDTA solubilized another 60% of the proteoglycans, while 33% still remained in the insoluble residue. Similar extraction yields have previously been obtained by direct extraction with 4M-guanidinium chloride containing EDTA (Engfeldt & Hjerpe, 1976). Corroborating previous data (Hjertquist & Vejlens, 1968), the major type of glycosaminoglycans in extracts and residue were galactosaminoglycans, i.e. chondroitin sulphate and/or dermatan sulphate. The first extract, as well

Table 1. *Distribution of proteoglycans in bone extracts and residue*

Ground bone tissue (50g wet wt.) was extracted with the two-step extraction procedure. For experimental details see the Materials and methods section. Means of three separate experiments are given. Proteoglycans are expressed as glycosaminoglycan-bound hexosamine (means ± S.E.M.).

Fractions	Glucosaminoglycan/galactosaminoglycan ratio (w/w)	Extraction yield (% hexosamines in extracts and residue)
Extract I (guanidine)	0.093	7.7±2.0
Extract II (guanidine/EDTA)	0.024	61.0±0.7
Residue	0.042	31.3±2.8

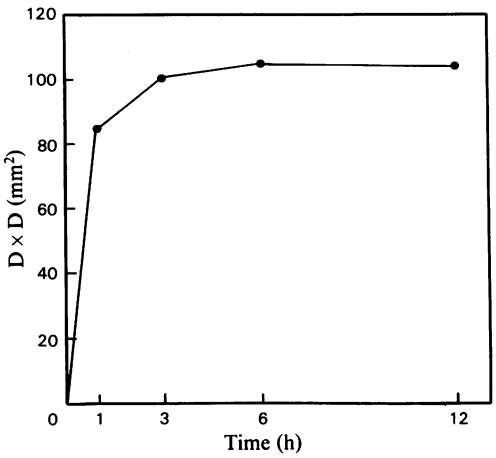


Fig. 1. *Extraction of 'non-specific' proteoglycans from bone preparation*

The diagram shows the time course of extraction from mature bovine bone of proteoglycans immunoreactive with antibodies to aorta proteoglycans. Ground bovine bone was extracted for various times at −20°C with 4M-guanidinium chloride containing proteinase inhibitors. The amount of extracted immuno-reactive proteoglycans was determined by a radial immunodiffusion by the method of Mancini *et al.* (1965), with equal-sized samples of the various bone extracts. D represents the diameter of the immunoprecipitates.

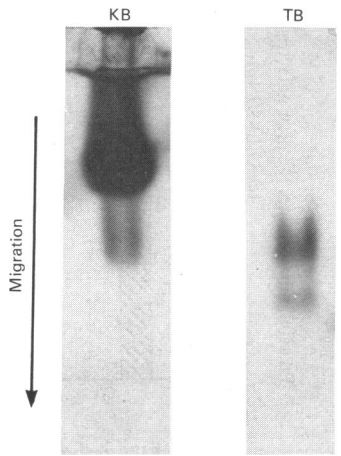


Fig. 2. *Analysis of bone extract containing 'specific' proteoglycans*

Measured samples of the second extract (4M-guanidinium chloride/EDTA) of bovine bone tissue were subjected to agarose/polyacrylamide-gel electrophoresis. The gels were stained with Kenacid Blue R (KB) for detection of proteins and with Toluidine Blue (TB) for detection of glycosaminoglycans. For further experimental details see the Materials and methods section.

as the residue, contained somewhat higher proportions of glucosaminoglycans than did the second extract, perhaps reflecting higher contents of heparan sulphate and hyaluronic acid.

The second extract was concentrated by ultrafiltration. Some organic material (about 30% of the total) was lost during this procedure. The glucosaminoglycan/galactosaminoglycan ratio, however, remained constant, indicating no selective loss of proteoglycans.

Samples of extract II were electrophoresed on an agarose/polyacrylamide gel after ethanol precipitation. Staining with Toluidine Blue showed two metachromatic bands (Fig. 2). The one with highest mobility had the same electrophoretic mobility as a reference of chondroitin 6-sulphate chains from nucleus pulposus. This component did not stain with Kenacid Blue, indicating a very low content of protein. It appears, then, that this fast-moving component represents glycosaminoglycan chains solubilized from the calcified bone matrix. The component with lower mobility showed both metachromasia and was stained with Kenacid Blue for protein. It had the same electrophoretic mobility as the small proteoglycans found in many other connective tissues, and could represent a proteoglycan of similar type.

The presence of glycosaminoglycan chains in extracts of connective tissues has not been described previously. Extreme care was taken to prevent proteolytic degradation during extraction, so the chains are most probably produced by a normal catabolic process. Whether the chains are derived from the type of proteoglycan present in the extract

or from other types of proteoglycans present during other stages of bone formation is not known. It is noteworthy, however, that when rat bone was extracted by using the same procedure only the proteoglycan component was found, with no identifiable faster-moving glycosaminoglycan chains (A. Franzén & D. Heinegård, unpublished work).

CsCl-density-gradient centrifugation

Proteoglycans were purified by CsCl-density-gradient centrifugation under dissociative conditions in 4M-guanidinium chloride, to minimize the effects of any proteinases present. The major portion of the proteoglycans and glycosaminoglycans

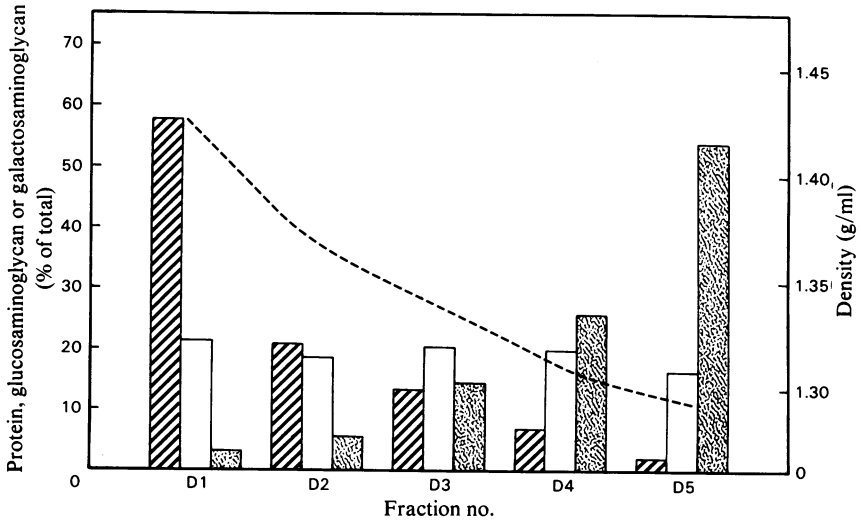


Fig. 3. *CsCl-density-gradient centrifugation of bone extract II*

The second extract (4M-guanidine/EDTA) of the ground bovine bone tissue was subjected to CsCl-density-gradient centrifugation. The centrifugation tubes were emptied from the bottom into five equal fractions, D1 (bottom) to D5 (top). Measured samples of the gradient fractions were analysed for content of proteins (▩), glucosaminoglycans (□) and galactosaminoglycans (▨). For experimental details see the Materials and methods section.

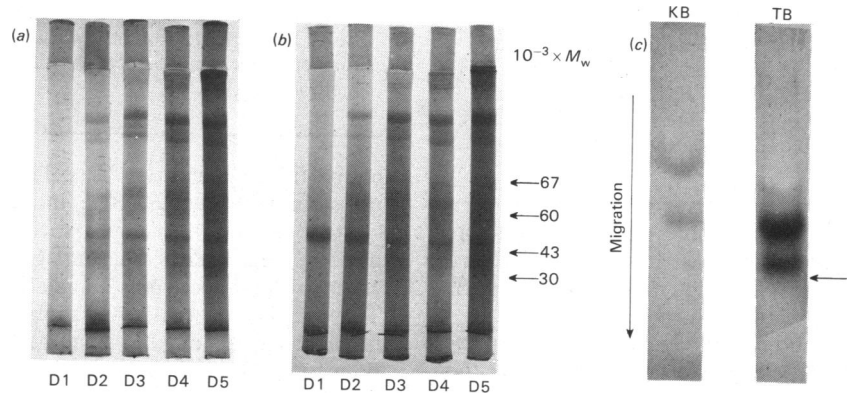


Fig. 4. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and agarose/polyacrylamide-gel electrophoresis of samples from the CsCl-gradient centrifugation of bone extract*

(*a*) and (*b*) Equal-sized samples from the CsCl-density-gradient fractions of the second bone extract were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Before electrophoresis the gradient fractions were incubated at 37°C for 6h without (*a*) and with (*b*) previous addition of chondroitinase ABC. Before electrophoresis the samples were reduced with 2-mercaptoethanol. For details see the Materials and methods section. (*c*) Equal-sized samples of the bottom fraction (D1) obtained from the CsCl-density-gradient centrifugation were subjected to agarose/polyacrylamide gel electrophoresis. The gel was stained with Kenacid Blue R (KB) or Toluidine Blue (TB). The horizontal arrow indicates the migration distance of the chondroitin 6-sulphate chains used as reference. For experimental details see the Materials and methods section.

was recovered at the bottom of the gradient, while most of the protein was found at the top (Fig. 3). The bottom D1 fraction ($\rho = 1.42$ g/ml) contained about 55% of the galactosaminoglycans present in the gradient. The relative proportion of glucosaminoglycans increased with decreasing buoyant density, indicating a changing proteoglycan composition (Fig. 3).

Equal-sized samples of the fractions were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with and without previous removal of galactosaminoglycan side chains by digestion with chondroitinase ABC, and this thereby reveals the molecular dimensions of the core protein preparation (Heinegård *et al.*, 1981). The bottom (D1) fraction contained a small amount of protein migrating with the front, whereas all other fractions contained several other protein components (Fig. 4). After chondroitinase digestion of the sample, however, fraction D1 contained a new component representing the proteoglycan protein core with an apparent M_r of 46000. Interestingly, proteoglycans that upon chondroitinase digestion yield core proteins with similar apparent M_r values on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis have been isolated from bovine nasal cartilage (Heinegård *et al.*, 1981), tendon (K. Vogel & D. Heinegård, unpublished work) and sclera (Cöster & Fransson, 1981). Fraction D2 contained a component with the same electrophoretic mobility, which although present before digestion became more pronounced after chondroitinase digestion (Fig. 4).

The D1 fraction was also characterized by agarose/polyacrylamide-gel electrophoresis. Two bands showed metachromasia with Toluidine Blue staining, but only the slow-moving component also stained for protein (Fig. 4) and therefore probably represents the proteoglycan.

Sepharose CL-4B chromatography

The bottom D1 fraction contained the major portion of the proteoglycans as shown by glycosaminoglycan contents and by agarose/polyacryl-

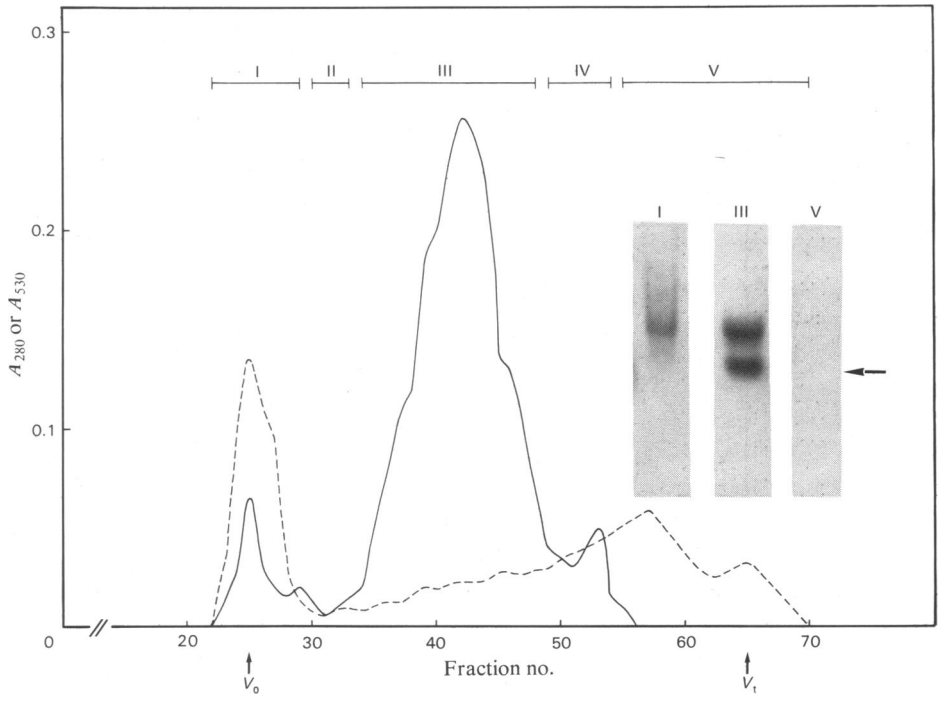


Fig. 5. *Sepharose CL-4B chromatography of partially purified bone proteoglycans*

The bottom fraction (D1) obtained from the CsCl-density-gradient centrifugation was subjected to Sepharose CL-4B chromatography in the presence of 4M-guanidinium chloride. The fractions were analysed for contents of protein (measured as A_{280}, ----) and hexuronic acid (carbazole reaction, A_{530}, ——). The eluate was pooled into five fractions (I–V) as indicated in the Figure. For further experimental details see the Materials and methods section. V_0, Void volume; V_t, total volume. Inset. Measured samples of the fractions I, III and V obtained from the Sepharose CL-4B chromatography were subjected to agarose/polyacrylamide-gel electrophoresis. After electrophoresis the gel was stained with Toluidine Blue for detection of glycosaminoglycans. The horizontal arrow indicates the migration of the standard (chondroitin 6-sulphate chains). The experiment details are further described in the Materials and methods section.

amide-gel electrophoresis. Furthermore, this fraction had a very low content of contaminating proteins and was therefore used as starting material for the purification of the proteoglycans by gel chromatography on Sepharose CL-4B eluted with 4M-guanidinium chloride. The elution pattern showed a void-volume peak, mainly containing nucleic acid as indicated by its high absorbance at 260nm (Fig. 5). The proteoglycans (about 90% of the glycosaminoglycans in the chromatogram) were eluted as a broad retarded peak, which contained more than one protein component. The colour yield in the carbazole reaction obtained with the void-volume component is probably non-specific, since it corresponded to only some 2% of the total glycosaminoglycan hexosamine.

Agarose/polyacrylamide-gel electrophoresis of equal-sized samples of the pooled fractions (Fig. 5 inset) showed that the void-volume peak contained three weakly staining components, with lower electrophoretic mobility than fraction III, possibly representing a small proportion of larger proteoglycans. The major fraction of proteoglycans (III) contained two components with mobilities corre-

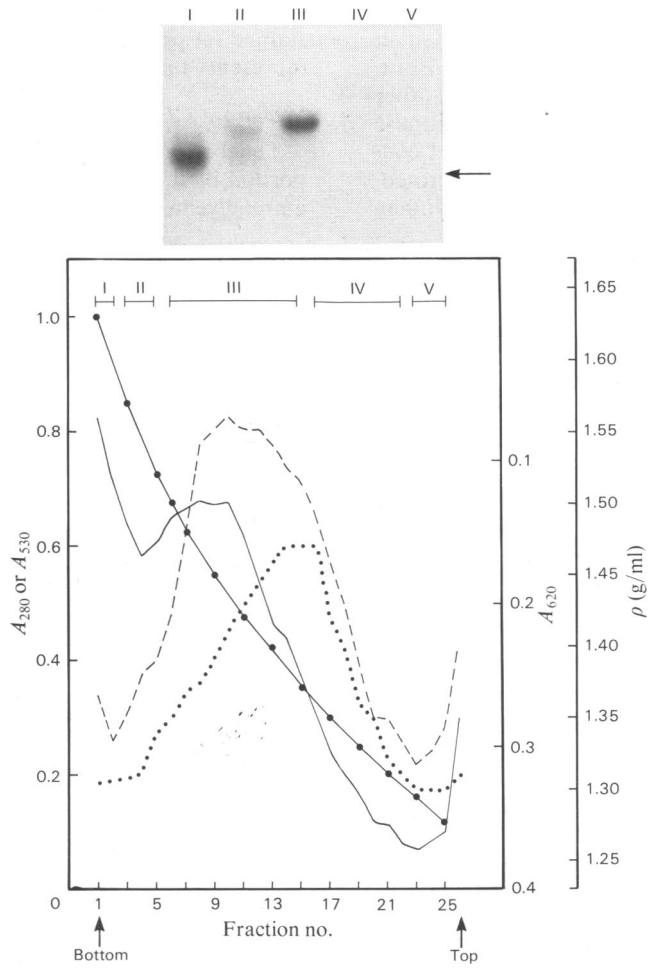


Fig. 6. *CsCl-density-gradient centrifugation in the presence of 4M-guanidinium chloride of fraction III obtained from the Sepharose CL-4B chromatography*

After centrifugation, the tube was emptied by pumping the solution from the bottom with a peristaltic pump. The density of every second fraction was determined by pycnometry (●—●). The fractions were analysed for content of protein (A_{280}, ----), hexuronic acid (A_{530}, ——) and sialic acid (A_{620}, ·····). The CsCl gradient was pooled into five (I–V) fractions as indicated in the Figure. For further details see the Materials and methods section. Inset. At the top is shown the agarose/polyacrylamide-gel electrophoresis of identical samples from the fractions (I–V) of the second CsCl-density-gradient centrifugation. The gel was stained with Toluidine Blue for detection of glycosaminoglycans. The horizontal arrow indicates the position of the chondroitin sulphate chains. For experimental details see the Materials and methods section.

sponding to small proteoglycans in cartilage (Heinegård *et al.*, 1981) and to chondroitin sulphate chains.

The fractions (I–V) were also electrophoresed on sodium dodecyl sulphate/polyacrylamide gels with and without prior chondroitinase ABC digestion as described above (results not shown). No proteins were observed in the gels corresponding to fractions I and II. Fraction III contained a major component that after digestion with chondroitinase had an apparent M_r of 46000. This component did not enter the gel if not digested with chondroitinase, and therefore represents the proteoglycan protein core. Fraction V contained protein migrating with the electrophoresis front. Since this protein was recovered in the high-density bottom fraction after CsCl-gradient centrifugation it is polyanionic, possibly representing phosphoprotein.

High-density CsCl-gradient centrifugation

A second CsCl-density-gradient centrifugation was used to separate the proteoglycans from the chondroitin sulphate chains. The partially purified proteoglycans (fraction III, Fig. 5) were further centrifuged in CsCl containing 4M-guanidinium chloride at a somewhat higher starting density (1.42g/ml) and higher centrifugation rate when compared with the initial centrifugation. The bottom, high-density, fractions contained a component rich in uronic acid and poor in protein (Fig. 6). It accounted for about 20% of the total hexuronic acid in the gradient and contained glycosaminoglycan chains and little or no proteoglycan, as shown by agarose/polyacrylamide-gel electrophoresis (Fig. 6 inset) and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after chondroitinase digestion (results not shown). The remainder of the uronic acid distributed in a broad peak extending over the bottom two-thirds of the gradient (Fig. 6).

The gradient fractions were also analysed for sialic acid contents. A component very rich in sialic acid and partially separated from the main hexuronic acid-containing peak was found in fractions of lower densities.

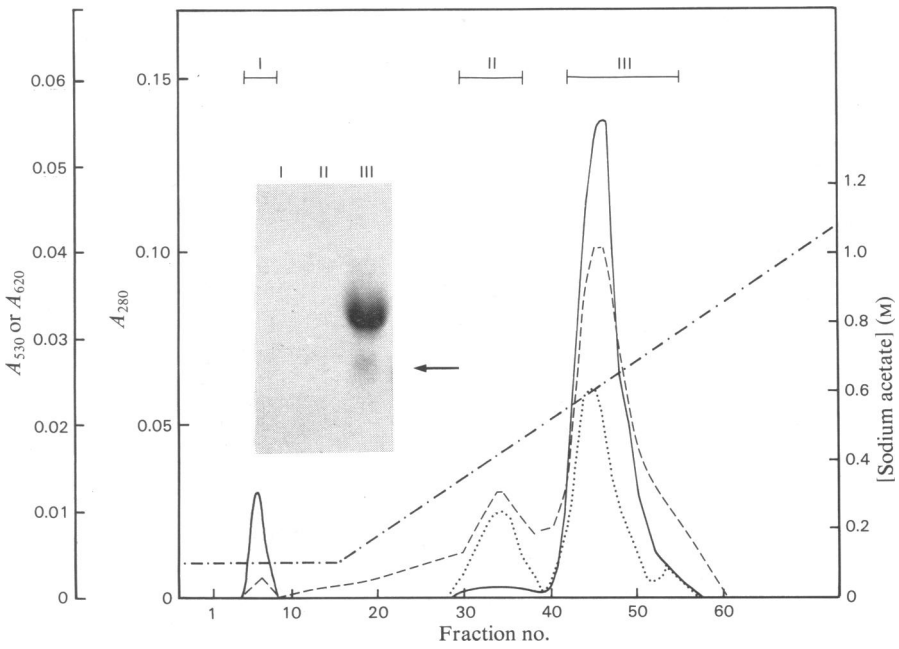


Fig. 7. *DEAE-cellulose (DE-52) ion-exchange chromatography in the presence of 7M-urea of fraction III obtained from the second CsCl-density-gradient centrifugation*

The fractions were analysed for content of protein (A_{280}, ----), hexuronic acid (A_{530}, ——) and sialic acid (A_{620}, ·····). The slope of the sodium acetate gradient (·–·–·) was determined by conductivity measurements, with standards of known sodium acetate concentration. The chromatogram was pooled into three fractions (I–III) as indicated in the Figure. The experimental protocol is described in the Materials and methods section. Inset. Measured samples of the fractions I–III obtained from the chromatogram were subjected to agarose/polyacrylamide-gel electrophoresis. The gel was stained with Toluidine Blue for detection of glycosaminoglycans. The horizontal arrow indicates the position of chondroitin sulphate chains.

Anion-exchange chromatography

An attempt was made to separate the sialic acid-containing component from the proteoglycan in the pooled fraction III of the CsCl gradient by using ion-exchange chromatography.

Fraction III was dialysed into 7M-urea/0.1M-sodium acetate buffer, pH6.0, and chromatographed on DEAE-cellulose (DE-52). Bound material was eluted with a linear gradient of sodium acetate from 0.1M to 1.2M. Two well-separated peaks were observed, the leading one being eluted at 0.45M-sodium acetate and apparently containing the sialic acid-rich component but no proteoglycan, since it contained no uronic acid and no proteoglycan was observed on agarose/polyacrylamide-gel electrophoresis (Fig. 7 inset). This sialoprotein was not further characterized. The second peak was eluted at 0.7M-sodium acetate and contained the proteoglycan (Fig. 7). Surprisingly, agarose/polyacrylamide-gel electrophoresis showed that it also contained some chondroitin sulphate chains (Fig. 7 inset), although no such chains could be identified in the material used for chromatography (Fig. 6 inset). It is possible that some interactions can only be dissociated in urea solutions.

Hydroxyapatite chromatography

The proteoglycans (DE52-III) were further fractionated into subclasses by chromatography on hydroxyapatite eluted in denaturing conditions of urea with a linear sodium phosphate gradient. Some protein-rich material was not bound to the hydroxyapatite matrix and was eluted from the column before the gradient was started (Fig. 8). The bound material chromatographed in two main fractions. One component was eluted in a sharp peak at about 0.1M-sodium phosphate. It accounted for about 50% of the hexuronic acid and about 25% of the protein (absorbance at 280nm) in the chromatogram (Fig. 8). The other fraction required higher salt concentration for elution and was more heterogeneous. It appeared to contain at least two components. Surprisingly, they had higher protein/hexuronic acid ratios than that observed for the leading component. All components bound to hydroxyapatite, i.e. fractions II, III and IV, contained proteoglycans, as shown by agarose/polyacrylamide-gel electrophoresis (Fig. 8 inset). Three fractions, HTP II, III and IV, were pooled as indicated in the Figure. From a typical chromatogram 8.0mg, 2.4mg and 2.7mg respectively were recovered.

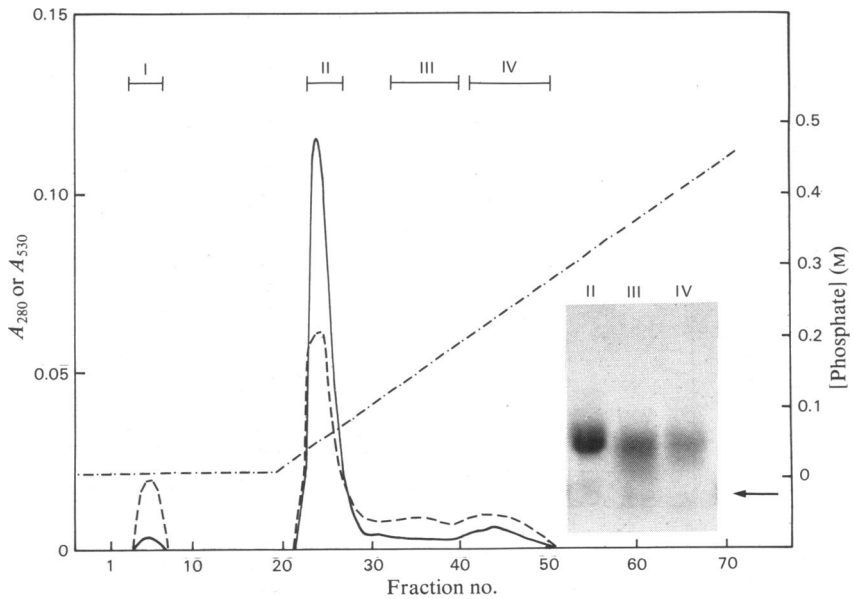


Fig. 8. *Hydroxyapatite chromatography in 7M-urea of fraction III from the DEAE-cellulose (DE-52) anion-exchange chromatography*

The column was eluted with a linear sodium phosphate gradient (·–·–·). The fractions were analysed for contents of protein (A_{280}, – – – –) and hexuronic acid (A_{530}, ——). The chromatogram was pooled into four fractions (I–IV) as indicated in the Figure. For experimental details see the Materials and methods section. Inset. Equal-sized samples of the fractions (I–IV) from the hydroxyapatite chromatography were subjected to agarose/polyacrylamide-gel electrophoresis. The gel was stained with Toluidine Blue to detect the glycosaminoglycans. The horizontal arrow indicates the position of chondroitin sulphate chains.

It should be stressed that chromatography on hydroxyapatite proved to be a prerequisite for removing small amounts of sialoprotein present in the proteoglycan fraction recovered from the DEAE-cellulose chromatography (the specific quantification of the sialoprotein by using an enzyme-linked immunosorbent assay is not included in this presentation). Interestingly, the sialoprotein showed higher affinity for the hydroxyapatite that did the proteoglycan.

Composition of the proteoglycan fractions

The analytical data indicate that the calcified matrix of bone contains at least three types of proteoglycans, i.e. fractions II, III and IV from the chromatography on hydroxyapatite, and additional chondroitin sulphate peptides, i.e. the high-density fraction (CsCl I) from the second CsCl gradient. These four fractions were further analysed (Table 2). The major proteoglycan fraction (HTP II) had a protein content of 39%. The predominating amino acids were leucine, aspartic acid/asparagine and glutamic acid/glutamine, indicating structural similarities with the small proteoglycans from other sources. Its hexosamine content was 14%, its uronic acid content 16.2% and its sialic content 0.8%. The major proportion of the hexosamines was galactosamine, indicating a high content of chondroitin sulphate.

The other two proteoglycan fractions had lower protein contents and also lower hexosamine contents compared with the major proteoglycan. Interestingly, their sialic acid contents were higher (Table 2). These two preparations had higher glutamic acid/glutamine and proline contents but

Table 2. *Amino acid and carbohydrate composition of the three bone proteoglycan populations (fractions HTP II, HTP III and HTP IV, Fig. 7) and the additional glycosaminoglycan peptide (fraction CsCl I, Fig. 5)*

	Composition (residues/1000 residues)			
	CsCl I	HTP II	HTP III	HTP IV
Hyp	Not detected			
Asp	125	128	126	145
Thr	53	45	52	66
Ser	100	76	102	106
Glu	160	105	158	205
Pro	79	77	72	54
Gly	114	86	114	126
Ala	48	49	53	45
Cys	Not determined			
Val	33	52	41	26
Met	Not determined			
Ile	56	58	36	27
Leu	81	128	83	46
Tyr	19	29	21	24
Phe	21	32	25	16
His	28	31	26	24
Lys	41	69	50	49
Arg	44	36	44	41
Protein* (% of dry wt.)	7.9	39.9	25.5	28.8
Hexuronic acid (% of dry wt.)	31.1	16.2	10.0	8.9
Sialic acid (% of dry wt.)	0.4	0.8	1.2	2.4
Hexosamines (% of dry wt.)	19.9	13.9	6.2	4.4

* From amino acid analysis.

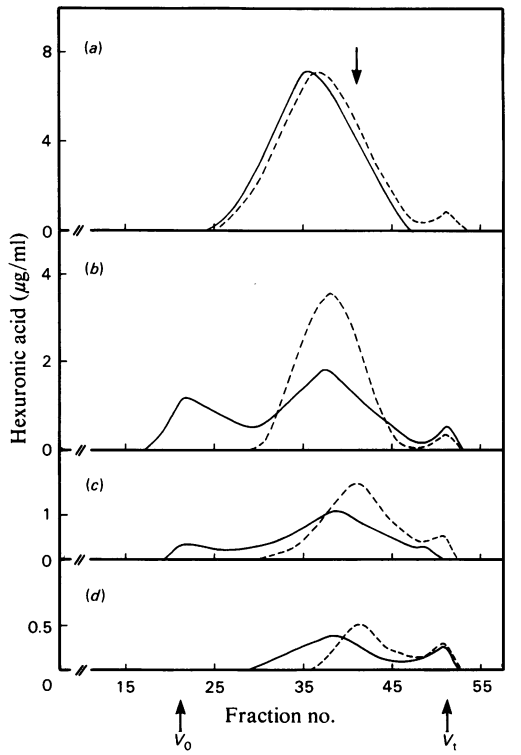


Fig. 9. *Gel chromatography of bone proteoglycan fractions and chondroitin sulphate peptides*

The various glycosaminoglycan-containing fractions isolated from the calcified matrix of bovine bone were subjected to analytical Sepharose 6B chromatography in 0.5M-sodium acetate buffer, pH 7.0. (*a*) CsCl I (bottom fraction, Fig. 6) and the fractions obtained from the hydroxyapatite chromatography; (*b*) HTP II; (*c*) HTP III; (*d*) HTP IV. The fractions were analysed for content of hexuronic acid of the intact molecules (——) and of the alkali-borohydride-treated molecules (----). The elution position of alkaline-borohydride-treated cartilage proteoglycan monomers is indicated with the arrow. For further experimental details see the Materials and methods section. V_0, Void volume; V_t, total volume.

contained less leucine than the major proteoglycan. Indeed, their amino acid composition was similar to that of the chondroitin sulphate peptides, although the latter expectedly had a very high hexosamine/protein ratio (Table 2). The high relative protein contents of fractions HTP III and HTP IV, however, makes it unlikely that these components represent degradation products of the major proteoglycan fraction.

Size distribution of the proteoglycan fractions

Chromatography on Sepharose 6B in 0.5M-sodium acetate, pH7.0, showed that the major component, i.e. HTP II, was eluted in two peaks (Fig. 9*b*), one in the void volume and the other well included. Since this material chromatographs in a well-included symmetrical peak under dissociating conditions of 4M-guanidinium chloride, it appears that the proteoglycans can form self-aggregates. Further proof was obtained by rechromatographing material from the two peaks, again under associative conditions. Both again gave rise to one void-volume peak and one included peak (results not shown).

The other two proteoglycan fractions chromatographed in major included peaks (Figs. 9*c* and 9*d*) corroborating their different nature. Fraction HTP III, however, gave a minor void-volume component, possibly representing some contamination with the major proteoglycan. Furthermore, alkaline-borohydride treatment to liberate the chondroitin sulphate side chains and subsequent gel chromatography gave further proof of the different nature of the proteoglycans. The major proteoglycan fraction, HTP II, contained side chains considerably larger ($K_{av.} = 0.52$) than those of the other two proteoglycan fractions ($K_{av.} = 0.67$), which in turn had side chains similar in size to those of major cartilage proteoglycans. The chondroitin sulphate chains in the chondroitin sulphate peptide fraction (CsCl I) had a size similar to that of the major proteoglycans (Fig. 9*a*), perhaps indicating that they are derived from these molecules by their normal turnover.

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