

# Isolation and characterization of two sialoproteins present only in bone calcified matrix

Ahnders FRANZÉN\*† and Dick HEINEGÅRD\*

\*Department of Physiological Chemistry, University of Lund, P.O. Box 94, S-221 00 Lund, Sweden, and †School of Dentistry, University of Lund, Carl Gustavs Väg 34, S-214 21 Malmö, Sweden

Two different sialoproteins were isolated from the mineralized matrix of bovine bone by using extraction with guanidinium chloride first without and then with EDTA. The sialoproteins were purified by chromatography on DEAE-cellulose eluted with a sodium acetate gradient in 7 M-urea, pH 6. Two sialoproteins (I and II) were then separated by chromatography on DEAE-cellulose eluted with a sodium chloride gradient in 7 M-urea, pH 4. The ratio between recovered sialoprotein I and II was 1:5. The chemical analysis of the two sialoproteins showed that they differed. Both, however, had very high contents of aspartic acid/asparagine and glutamic acid/glutamine though they differed markedly in contents of leucine and glycine. Both sialoproteins contained phosphate, sialoprotein I more than sialoprotein II. Content of sialic acid was substantially higher in the more prominent sialoprotein II (13.4% of dry weight) than in sialoprotein I (4.8% of dry weight). The peptide patterns produced by trypsin digests of [<sup>125</sup>I]iodinated sialoproteins I and II showed both structural similarities and structural differences. Sialoprotein II, being the major component, was characterized further. Its molecular mass was 57300 Da determined by sedimentation-equilibrium centrifugation in 6 M-guanidinium chloride, and its sedimentation coefficient ( $s_{20,w}^0$ ) was 2.53 S. Upon rotary shadowing, sialoprotein II appeared as an extended rod, having a core with an average length of 40 nm. Two types of oligosaccharides, *N*-glycosidically and *O*-glycosidically linked to the core protein, were isolated from sialoprotein II. Contents of mannose and sialic acid in the *O*-linked oligosaccharide were surprisingly high. Antibodies against sialoprotein II were raised in rabbits and an enzyme-linked immunosorbent assay was developed. Antigenicity of sialoprotein II was not affected by reduction and alkylation, was only partially lost upon trypsin digestion and was completely lost upon fragmentation of the core protein by alkaline-borohydride treatment, indicating that all antigenic sites were located in the protein portion. Sialoprotein I expectedly showed only partial immunological cross-reactivity with sialoprotein II. The quantity of sialoprotein II in bone extracts was found to be about 1.5 mg/g wet wt. of bone, but the protein was not detected in extracts of a number of other bovine tissues i.e. aorta, cartilage, dentine, kidney, liver, muscle, sclera, skin and tendon.

## INTRODUCTION

The organic matrix of bone contains a number of different types of macromolecules, where type I collagen represents as much as some 90% of the dry weight (Miller & Martin, 1968). Several other components have been identified, many being polyanionic, such as phosphoproteins (Spector & Glimcher, 1972), proteoglycans (Herring, 1968; Fisher *et al.*, 1983a; Franzén & Heinegård, 1983, 1984a,b) and sialoproteins (Herring & Kent, 1963; Fisher *et al.*, 1983b). Others, present only in bone, are not polyanionic, i.e. osteonectin (Termin *et al.*, 1981b) and osteocalcin (Hauschka *et al.*, 1975). Still others, such as albumin and  $\alpha$ 2-HS-glycoprotein, are of plasma origin (Ashton *et al.*, 1976).

Over the years much work has been focused on the sialic acid-rich proteins, which appear to be present only in bone (Herring, 1976; Fisher *et al.*, 1983b). This group of molecules was originally identified by Herring & Kent (1963), and much of the original characterization was done by Herring and co-workers. With improved technology allowing the isolation of more-intact molecules, Fisher *et al.* (1983b) isolated a sialic acid-rich protein of higher apparent molecular mass from extracts

of bovine foetal bone. From extracts of the mineralized matrix of rat bone we were able similarly to isolate sialic acid-rich proteins and furthermore to separate two different species of the proteins. Both sialoproteins were synthesized in explant cultures of rat calvaria (Franzén & Heinegård, 1985). The amounts of material available, however, were too small for extensive characterization of the two proteins.

The present investigation describes the purification and characterization of two species of sialoproteins from the mineralized matrix of bovine bone. With the use of immunoassay it is shown that the proteins are present only in bone.

## MATERIALS AND METHODS

### Chemicals

Ion-exchange DEAE-cellulose (DE52) was obtained from Whatman Chemicals, Maidstone, Kent, U.K. Sephacryl S-300, Sephadex G-50 and molecular-mass calibration kit were products of Pharmacia Fine Chemicals, Uppsala, Sweden, and Bio-Gel P-10 was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Alkaline

phosphatase substrate (*p*-nitrophenyl phosphate) and 1-chloro-4-phenyl-3-tosylamidobutan-2-one- ('TPCK') treated trypsin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

### Isolation of sialoproteins

**Extraction.** Frozen bone powder was extracted sequentially with 10 vol. of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, and 30 vol. of 4 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.4, containing 0.25 M-disodium EDTA as described in detail elsewhere (Franzén & Heinegård, 1984a). Both extraction solvents were also supplemented with proteinase inhibitors (Oegema *et al.*, 1975) and with 5 mM-*N*-ethylmaleimide, the latter included mainly to prevent disulphide-bond exchange (Heinegård *et al.*, 1981). Only the second extract (guanidinium chloride/EDTA), from the mineral phase, was analysed further. This extract was concentrated at 4 °C by ultrafiltration over a PM-10 filter (Amicon Corp., Lexington, MA, U.S.A.). The retained material was brought into 7 M-urea/0.1 M-sodium acetate/10 mM-Tris/HCl buffer, pH 6.0, by diaflow with 20 vol. of the urea solution.

**Anion-exchange chromatography at pH 6.** The guanidinium chloride/EDTA extract, brought into the 7 M-urea/Tris buffer (see above), was chromatographed on a DEAE-cellulose (DE52) ion-exchange column (1.2 cm × 9 cm) eluted at 4 °C with 7 M-urea/0.1 M-sodium acetate/10 mM-Tris/HCl buffer, pH 6, at a flow rate of 10 ml/h. Material not bound to the column was first eluted with 3 bed volumes of the urea/Tris buffer used for diaflow and then with a linear gradient of sodium acetate (0.1–1.2 M) in the urea/Tris buffer described above. Fractions (3 ml) were collected and analysed for contents of protein ( $A_{280}$ ) and sialic acid.

**Anion-exchange chromatography at pH 4.** The sialic acid-enriched component (fraction III) from the DEAE-cellulose chromatography at pH 6 was concentrated by ultrafiltration over a PM-10 filter (Amicon) and subsequently brought into 7 M-urea/10 mM-NaCl/50 mM-sodium acetate/10 mM-Tris/HCl buffer, pH 4, by diaflow. The sample was applied to a DEAE-cellulose (DE52) ion-exchange column (0.9 cm × 8 cm), which was first eluted at 20 °C with 3 bed volumes of 7 M-urea/10 mM-NaCl/50 mM-sodium acetate/10 mM-Tris/HCl buffer, pH 4, at a flow rate of 10 ml/h. The concentration of NaCl in the eluent was then increased to 50 mM, 6 bed volumes being used for elution. Finally, remaining material was eluted with a 0.05–0.5 M-NaCl linear gradient in 7 M-urea/50 mM-sodium acetate/10 mM-Tris/HCl buffer, pH 4. Fractions (2 ml) were collected and analysed for contents for protein ( $A_{280}$ ) and sialic acid. Two components enriched in sialic acid were pooled, dialysed against distilled water, freeze-dried and stored at –20 °C until analysed further.

### Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The samples to be electrophoresed were precipitated with 9 vol. of ethanol (Paulsson *et al.*, 1983) to remove traces of salts. Before electrophoresis the precipitates were freeze-dried and dissolved in aq. 1% (w/v) sodium dodecyl sulphate, mixed with an equal volume of double-concentrated electrophoresis buffer containing

2% (w/v) 2-mercaptoethanol and 2% (w/v) sodium dodecyl sulphate and incubated at 37 °C for 2 h. The samples were then electrophoresed on polyacrylamide slab gels ( $T = 8\%$ ,  $C = 2.5\%$ ) in the discontinuous buffer system described by Neville (1971). After electrophoresis the gels were stained with 0.25% (w/v) Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.).

### Chemical methods

The hydroxyproline content was determined by the method of Stegeman & Stalder (1967), and the content of inorganic phosphate was quantified after the samples had been ashed with conc.  $H_2SO_4$  as described by Chen *et al.* (1956). The amino acid composition was determined after hydrolysis of samples in 6 M-HCl (AristaR) at 110 °C for 24 h, and hexosamines were determined after hydrolysis in 4 M-HCl at 100 °C for 10 h in tubes sealed under argon. The protein content was calculated from the sum of amino acids. Sialic acid content was determined by using an automated version (Lohmander *et al.*, 1980) of the resorcinol procedure described by Jourdain *et al.* (1971). Before quantification of sialic acid, samples containing urea were diluted with distilled water to about 0.4 M to increase the colour yield.

Sialoproteins I and II were  $^{125}I$ -labelled by using the chloramine-T procedure as described elsewhere (Paulsson & Heinegård, 1982).

### Analysis of peptide patterns

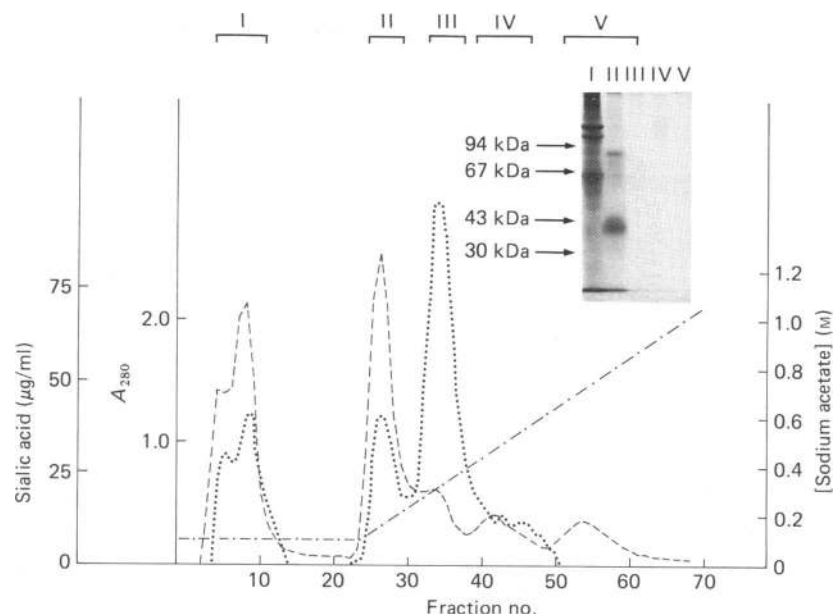
The labelled sialoproteins were dissolved in 0.1 M-Tris/HCl buffer, pH 8, and digested for 4 h at 37 °C with trypsin (1  $\mu$ g/ $\mu$ g of sample). The digests were subjected to high-voltage electrophoresis at pH 6.4 on silica plates (Merck, Darmstadt, West Germany). After drying, the plates were chromatographed, the second dimension being developed with butanol/pyridine/acetic acid/water (6:5:3:1, by vol.) as described by Bates & Perham (1975). The radioactivity was recorded by autoradiography for 2–3 days on Agfa-Gevaert Osray M3 X-ray films.

### Physicochemical characterization

**Gel chromatography.** Samples (0.5 mg) of sialoproteins I and II were dissolved in 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, and chromatographed on a Sephacryl S-300 column (0.6 cm × 140 cm) eluted with the same solvent.

**Determination of partial specific volume.** Sialoprotein II (23.7 mg) was dissolved in 6 M-guanidinium chloride/5 mM-Tris/HCl buffer, pH 7.4 (concn. 9.98 mg/ml, based on anhydrous weight) and dialysed against 50 ml of the buffer described above. The density of the sample was determined with a Kratky mechanical oscillator (model DM 60; A. P. Paar), and the partial specific volume was calculated by the method of Kratky *et al.* (1973).

**Analytical ultracentrifugation.** The molecular mass of sialoprotein II was determined by sedimentation-equilibrium centrifugation in 6 M-guanidinium chloride by using the meniscus-depletion technique described by Yphantis (1964). A sample of the preparation of sialoprotein II used for determination of partial specific volume was dissolved (5 mg/ml) in 6 M-guanidinium chloride/5 mM-Tris/HCl buffer, pH 7.4, and dialysed against a 30-fold excess of the solution. Centrifugation was done in an MSE Centriscan analytical ultracentrifuge



**Fig. 1. DEAE-cellulose (DE52) chromatography of guanidinium chloride/EDTA extract of mineralized bone matrix**

The second extract (i.e. guanidinium chloride/EDTA) of powdered diaphysal bovine bone was brought into 7 M-urea/0.1 M-sodium acetate/10 mM-Tris/HCl buffer, pH 6, and chromatographed on a DEAE-cellulose (DE52) column at 4 °C, with a flow rate of 10 ml/h. After elution with several volumes of starting buffer, the column was eluted with a linear gradient (0.1–1.2 M) of sodium acetate in 7 M-urea/buffer, pH 6 (— · — · —). Fractions were analysed for content of protein (— · — · —,  $A_{280}$ ) and sialic acid (· · · · ·,  $\mu\text{g/ml}$ ). The chromatogram was pooled into five (I–V) fractions as indicated. Samples representing equal proportions of fractions I–V were electrophoresed on sodium dodecyl sulphate/8% polyacrylamide gels (inset). Experimental details are given in the Materials and methods section.

at 23000 and at 25000 rev./min at 20 °C. Samples to be centrifuged were diluted from a dialysed stock solution by adding weighed samples of the dialysis residue to give final concentrations of 0.095 mg/ml, 0.190 mg/ml, 0.285 mg/ml and 0.380 mg/ml. Fluorocarbon oil (FC 70; 3M, Minneapolis, MN, U.S.A.) was added to the centrifuge cell to permit measurements in the bottom segment of the cell also. The establishment of sedimentation-equilibrium conditions were determined by repeatedly monitoring the distribution of the absorbance at 280 nm. Sedimentation-rate centrifugation in 6 M-guanidinium chloride was done at 55000 rev./min at 20 °C with samples of sialoprotein II diluted from the dialysed stock solution to concentrations of 1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml. The sedimentation characteristics of sialoprotein II were monitored by using schlieren optics and with scans taken at regular time intervals.

#### Characterization of oligosaccharide side chains

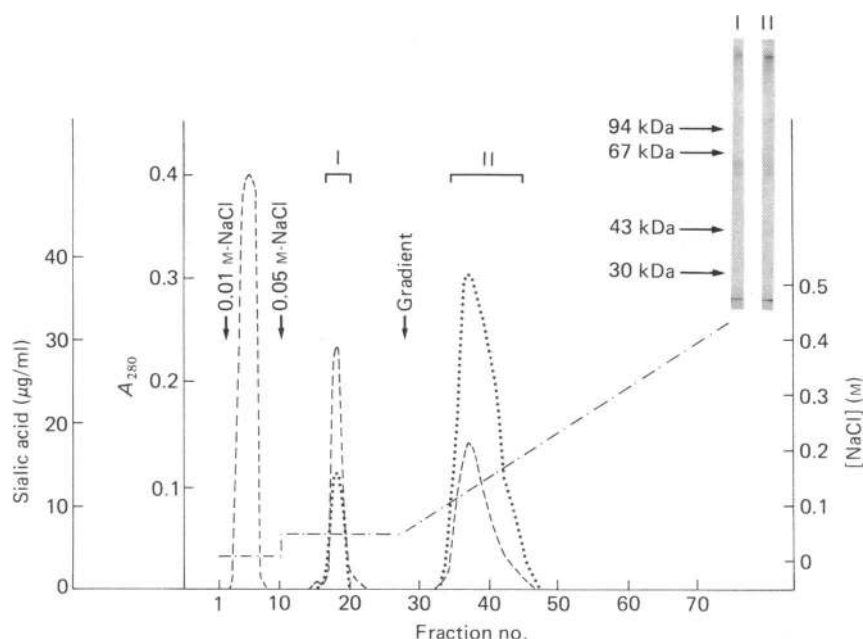
Oligosaccharides were liberated from sialoprotein II by alkaline-borohydride treatment as described by Carlson (1968). A sample (1.02 mg) of sialoprotein II was dissolved (5 mg/ml) in 50 mM-NaOH/1 M-NaBH<sub>4</sub> and incubated at 45 °C for 48 h. The alkaline-borohydride digest was then neutralized with 10 M-acetic acid, freeze-dried, dissolved in 1 M-pyridine/acetate buffer, pH 7.0, and chromatographed on a Bio-Gel P-10 column (0.6 cm × 140 cm) eluted at 20 °C with 1 M-pyridine/acetate buffer, pH 7.0. The fractions obtained were freeze-dried to remove pyridine/acetate, dissolved in distilled water and analysed for contents of sialic acid (see above under 'Chemical methods'). The chromatogram was pooled to give two fractions (I and II). Measured

samples of these fractions were analysed for hexosamine content (as described above under 'Chemical methods'). Other samples were used for the determination of neutral-sugar composition after hydrolysis in 2 M-trifluoroacetic acid for 3 h at 100 °C. Monosaccharides were separated on a Bio-Rad HPX-87P carbohydrate column (Bio-Rad Laboratories) eluted with water. The effluent was analysed for carbohydrates by using an automated version of the orcinol method (S. Lohmander, personal communication).

#### Immunology

**Preparation of antibodies.** Antibodies directed against sialoprotein II were raised in rabbits. Initial immunization was done with 1 mg of sialoprotein II in Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.). Booster doses with 0.5 mg of sialoprotein II in Freund's incomplete adjuvant (Difco Laboratories) were given monthly until the antibody titre was sufficient.

**Enzyme-linked immunosorbent assay.** Sialoprotein II, dissolved at 1  $\mu\text{g/ml}$  in 4 M-guanidinium chloride/10 mM-sodium phosphate buffer, pH 7.4, was used to coat the wells of a poly(vinyl chloride) micro-titre plate (Dynatech M29) by incubation overnight at 4 °C. The plates were then rinsed extensively with PBS/Tween (0.15 M-NaCl/5 mM-sodium phosphate buffer, pH 7.4, containing 0.05% Tween 20). Dilutions of purified sialoprotein II and samples to be analysed, made in PBS/Tween, were pre-incubated at 4 °C for 18 h with an equal volume of antibodies against sialoprotein II diluted at 1:2000 with PBS/Tween. Measured samples of the antigen/antibody mixture were added to the wells coated with sialoprotein II,



**Fig. 2. DEAE-cellulose (DE52) chromatography of partially purified sialoproteins**

The fraction obtained from DEAE-cellulose chromatography at pH 6 (Fig. 1) containing sialoproteins (DE52 III) was dialysed into 7 M urea/10 mM-NaCl/50 mM-sodium acetate/10 mM-Tris/HCl buffer, pH 4, and chromatographed on a DEAE-cellulose column, eluted at 20 °C at a flow rate of 5 ml/h. The column was eluted as illustrated, i.e. by a combination of stepwise elutions (10 mM- and 50 mM-NaCl) and with a linear gradient (0.05–0.5 M) of NaCl in the 7 M-urea/buffer, pH 4 (---), described above. The contents of protein (—,  $A_{280}$ ) and sialic acid (·····,  $\mu\text{g/ml}$ ) were determined. The two components containing sialic acid, sialoprotein I and sialoprotein II, were pooled as indicated in the chromatogram. Identical samples of sialoproteins I and II were electrophoresed on sodium dodecyl sulphate/8% polyacrylamide gels (inset). Experimental details are given in the Materials and methods section.

and the plates were incubated for 1 h at 20 °C. After a rinsing with PBS/Tween, bound antibodies were detected by incubation with a dilution (1:200 in PBS/Tween supplemented with 2 mg of bovine serum albumin/ml) of pig anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase (Orion Diagnostica, Helsinki, Finland). After incubation for 1 h at 20 °C, the wells were rinsed and enzyme activity was measured by incubation with *p*-nitrophenyl phosphate (1 mg/ml) in 1 M-diethanol-amine/0.5 mM-MgCl<sub>2</sub>, pH 9.8. The absorbance at 405 nm of liberated *p*-nitrophenol was measured with a Titertec Multiscan filter photometer (Flow Laboratories, Rockville, MD, U.S.A.).

#### Distribution of sialoprotein II among bovine tissues

Bovine aorta, diaphysial bone, articular cartilage, dentine, kidney, liver, muscle, sclera, skin and tendon were obtained from the local slaughterhouse. The tissues were powdered in liquid N<sub>2</sub> and extracted for 24 h at 4 °C with 30 ml/g of 4 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.4, supplemented with 0.25 M-disodium EDTA, proteinase inhibitors (Oegema *et al.*, 1975) and 5 mM-*N*-ethylmaleimide. Samples of the extracts were transferred into 7 M-urea/0.1 M-sodium acetate/10 mM-Tris/HCl buffer, pH 6, by chromatography on Sephadex G-50 columns (0.8 cm × 20 cm) equilibrated with the urea/buffer. The fractions eluted in the void volume were pooled and subsequently chromatographed on DEAE-cellulose columns equilibrated with the urea/buffer described, and eluted stepwise with 3 vol. (= 3 ml) of 0.3 M- and 0.7 M-sodium acetate respectively,

to elute the sialoproteins. Fractions representing material bound to the column were analysed for content of sialoprotein II by using an enzyme-linked immunosorbent inhibition assay.

## RESULTS AND DISCUSSION

In the present study two different sialoproteins were isolated from the mineralized matrix of bovine diaphysial bone. Macromolecules present in the non-mineralized tissues of the bone, i.e. those in blood vessels, bone marrow cells and fibrous tissues, were initially extracted from powdered bone with 4 M-guanidinium chloride containing proteinase inhibitors. Remaining extractable molecules present in the mineralized bone matrix were then solubilized when 0.25 M-EDTA was included in the extraction solvent to dissolve the bone mineral (i.e. hydroxyapatite crystals).

#### Purification of sialoproteins

The guanidinium chloride/EDTA extract was brought into 7 M-urea/0.1 M-sodium acetate buffer, pH 6, and chromatographed on DEAE-cellulose (Fig. 1). The molecules bound to the column were eluted with a linear gradient of 0.1–1.2 M-sodium acetate in 7 M-urea/buffer, pH 6. Five peaks having absorbance at 280 nm were identified in the chromatogram, and four of these also contained sialic acid. More than 70% of the absorbance at 280 nm was equally distributed among the two leading peaks, one peak eluted before and the other shortly after the start of the gradient. These two peaks had similar

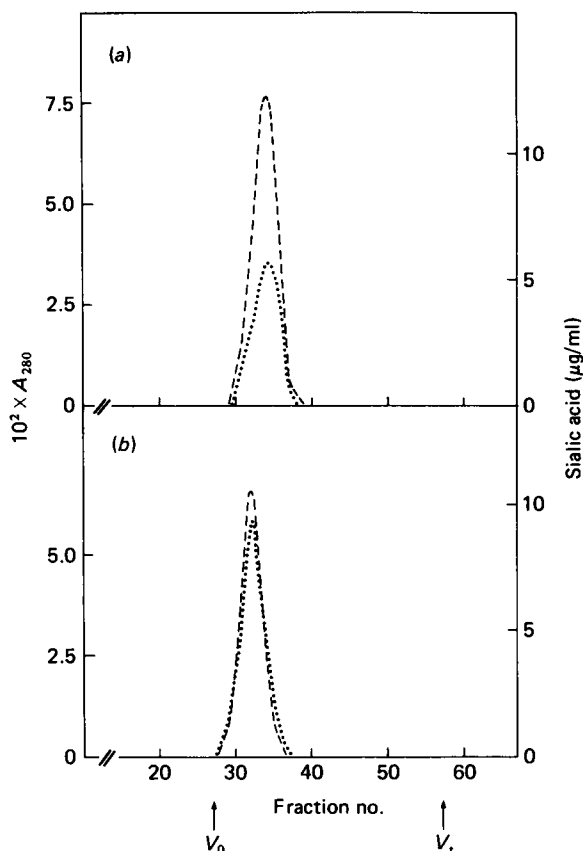


Fig. 3. Sephacryl S-300 gel chromatography of sialoproteins I and II

Samples (0.5 mg) of sialoprotein I (a) and sialoprotein II (b) were dissolved in 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, and chromatographed on a column (0.6 cm  $\times$  140 cm) of Sephacryl S-300 eluted with the same solvent. Fractions were analysed for contents of proteins (----,  $A_{280}$ ) and sialic acid (.....,  $\mu\text{g/ml}$ ). Experimental details are given in the Materials and methods section.

ratios of sialic acid to protein. A third peak with a high sialic acid/protein ratio accounted for more than 50% of the sialic acid present in the chromatogram. It was eluted at about 0.4 M-sodium acetate. The fourth component, which was eluted at about 0.6 M-sodium acetate, contained less protein and sialic acid when compared with the other components, but had a high content of hexuronic acid (detailed results not shown). The component that was eluted most retarded in the chromatogram, at about 0.75 M-sodium acetate, did not contain any sialic acid and had a high  $A_{260}/A_{280}$  ratio (detailed results not shown). This material most probably represents nucleic acid. The chromatogram was pooled in five (I–V) fractions as indicated in Fig. 1. Macromolecules in samples representing the same proportion of the total were precipitated with 9 vol. of ethanol and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels (Fig. 1 inset). The leading peak (fraction I) contained a large number of proteins, but fraction II contained fewer, i.e. four or five proteins where the one predominating had an apparent molecular mass of 43 000 Da. This component was later identified as osteonectin (Termine *et al.*, 1981b), by using antibodies

directed against calf osteonectin (kindly supplied by Dr. John D. Termine, National Institutes of Health, Bethesda, MD, U.S.A.). Another main component in this fraction had an apparent molecular mass of 85 000 Da. Fraction III contained only one or two very weakly stained proteins, which most probably are sialoproteins, related to those originally described by Herring & Kent (1963). Fraction IV contained one component, with an apparent molecular mass of about 120 000 Da. It was

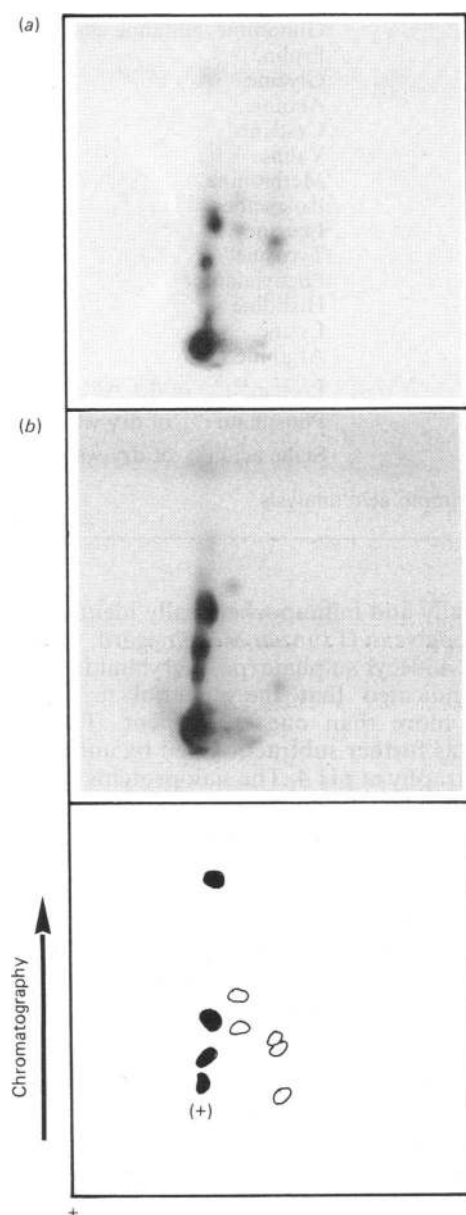


Fig. 4. Peptide patterns produced by trypsin digestion of [ $^{125}\text{I}$ ]iodinated sialoprotein I (a) and sialoprotein II (b)

The sialoproteins were labelled with  $^{125}\text{I}$  by using the chloramine-T procedure and digested with trypsin. Samples of the digests were applied to silica-gel thin-layer plates and separated in the first dimension by electrophoresis and in the second by chromatography. The application point (+) is indicated. Experimental details are given in the Materials and methods section. The bottom panel shows all identified peptides, the blackened spots representing peptides found in both proteins.

Table 1. Amino acid compositions and contents of protein, phosphate and sialic acid in sialoproteins I and II

	Amino acid composition (mol of residue/1000 mol of residues)	
	Sialoprotein I	Sialoprotein II
Hydroxyproline	Not detected	Not detected
Asparagine/aspartic acid	137.3	165.8
Threonine	58.5	83.5
Serine	43.7	95.3
Glutamine/glutamic acid	167.5	204.3
Proline	73.6	58.1
Glycine	72.3	113.2
Alanine	58.9	53.2
Cysteine	Not determined	Not determined
Valine	59.8	32.6
Methionine	Not determined	Not determined
Isoleucine	35.6	26.6
Leucine	88.2	36.0
Tyrosine	23.8	37.3
Phenylalanine	38.7	20.5
Histidine	41.1	9.0
Lysine	67.2	39.1
Arginine	33.9	26.2
Protein* (% of dry wt.)	45.3%	39.9%
Phosphate (% of dry wt.)	3.5%	0.7%
Sialic acid (% of dry wt.)	4.8%	13.4%

\* From amino acid analysis.

biochemically and immunochemically identical with the bone proteoglycan (Franzén & Heinegård, 1984b).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated that the material in fraction III contained more than one component. Therefore the material was further subfractionated by anion-exchange chromatography at pH 4. The sialoproteins (fraction III, Fig. 1) were brought into 7 M-urea/10 mM-NaCl/50 mM-sodium acetate/10 mM-Tris/HCl buffer, pH 4, and chromatographed on a DEAE-cellulose ion-exchange column. Material not bound to the column was eluted with 10 mM-NaCl, and components bound to the column were first eluted with 50 mM-NaCl and then with a gradient of NaCl (0.05–0.5 M), all eluents containing 7 M-urea/buffer as described above. The material was separated into three different components (Fig. 2). One component, which did not bind to the column, showed a high absorbance at 280 nm. Since it did not contain any sialic acid, it was not analysed further. A component enriched in sialic acid, sialoprotein I, was eluted in a very sharp peak when the NaCl concentration was increased to 50 mM. Sialoprotein II was eluted at about 0.1 M-NaCl with the gradient. The sialic acid/protein ratio in sialoprotein I was only about one-half of that of sialoprotein II. The chromatogram was pooled to give two fractions, as indicated in Fig. 2. Samples of the two preparations were electrophoresed on a sodium dodecyl sulphate/8% polyacrylamide gel (Fig. 2 inset). One weakly stained component was identified in each sample. The absence of other stainable proteins indicated that the two preparations contain pure sialoproteins. The purity of the two sialoproteins was further checked by gel chromatography of the two preparations on Sephacryl S-300 (Fig. 3). Each preparation contained only one

symmetrical peak, the one from sialoprotein II being eluted somewhat less retarded, showing that this molecule is somewhat larger. By using the same purification scheme two species of sialoproteins have recently been isolated from human bone (A. Franzén D. Heinegård, unpublished work) and from rat bone (Franzén & Heinegård, 1985). Fisher *et al.* (1983b) prepared a sialoprotein of very similar character and size from foetal calf bone. They did not, however, subfractionate the preparation to yield subpopulations. Originally Herring and co-workers (for review see Herring, 1972) isolated a protein with a very high content of sialic acid from bone extracts. They named this protein sialoprotein. Its molecular mass was about 25000 Da (Williams & Peacocke, 1965), i.e. much lower than that of the molecules discussed in the present work. Whether or not that preparation represents the same molecule as the one described presently is not fully answered by the data available. The previously used long extraction times and the use of extraction solvents not containing denaturing solvents and proteinase inhibitors is known to result in fragmentation of bone proteins (Termine *et al.*, 1981a). Therefore it is likely that the early described preparations represent fragments of the native sialoproteins characterized in the present work. In support of this, the sialic acid contents of the presently described molecules are somewhat lower. Furthermore, the amino acid compositions of early preparations have similarities with those presented in this work, with very high contents of aspartic acid/asparagine and glutamic acid/glutamine.

One problem in comparing the preparation obtained with the presently used technique with those originally prepared is that we find that, by eluting the ion-exchange columns with solutions containing 7 M-urea, yields were

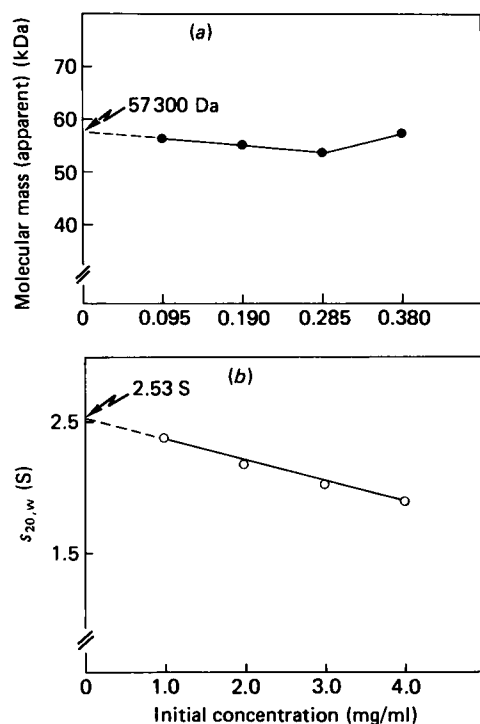


Fig. 5. Analytical ultracentrifugation of sialoprotein II

(a) Determination of molecular mass of sialoprotein II by sedimentation-equilibrium centrifugation; (b) determination of sedimentation coefficient of sialoprotein II by sedimentation-rate centrifugation. The centrifugations were performed in 6 M-guanidinium chloride/5 mM-Tris/HCl buffer, pH 7.4, and the distribution of sedimenting material was analysed by monitoring  $A_{280}$  (●) or by using schlieren optics (○). Further information about the experiments is given in the Materials and methods section.

improved from about 20% to better than 90% (A. Franzén & D. Heinegård, unpublished work). It is possible that in the early work losses of the large intact sialoproteins were more pronounced, since urea was not used.

### Comparison of sialoproteins I and II

The two sialoproteins, which were recovered in a mass ratio of 1:5 (I/II), were compared with respect to composition (Table 1) and to peptide patterns. The protein content (percentage of dry weight) of sialoproteins I and II was 45% and 40%, respectively. The predominating amino acids, together accounting for one-third of the residues in each of the two sialoproteins, were aspartic acid/asparagine and glutamic acid/glutamine. The leucine content was higher in sialoprotein I, and sialoprotein II showed a higher content of glycine. Sialic acid accounted for 4.8% of the dry weight of sialoprotein I and for as much as 13.4% of the dry weight of sialoprotein II. Sialoprotein I had a more than 6-fold higher content of phosphate (4.5% of dry weight) compared with sialoprotein II (0.7% of dry weight). Neither of the molecules, however, appear to be related to the phosphoproteins isolated from bone (Termine *et al.*, 1981a), since their composition is distinctly different. The two species of sialoproteins isolated from rat bone also showed differences in phosphate content (Franzén & Heinegård,

1985) of the same order. On the other hand, sialoproteins I and II from rat bone had almost the same content of sialic acid, in both cases accounting for about 4.5% of the dry weight (Franzén & Heinegård, 1985).

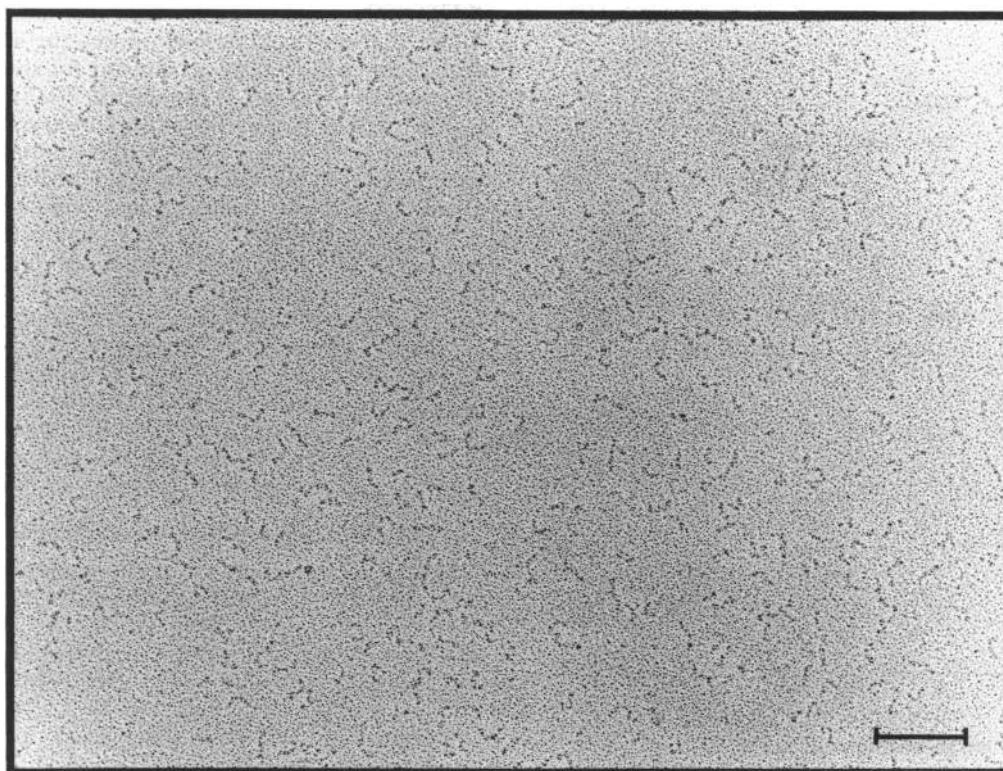
The peptide patterns produced by trypsin digestion of [ $^{125}$ I]iodinated sialoprotein I and sialoprotein II were compared (Fig. 4). The same main labelled peptides were present in both preparations, indicating structural similarities between the two sialoproteins. A few peptides, however, were unique for one or the other protein, indicating that the two sialoproteins represent different gene products. It appears that the protein eluted at lower ionic strength belongs to the same family of sialoproteins, although it has a considerably lower sialic acid content and contains phosphate. Its amino acid composition and protein backbone are similar to that of the major sialoprotein, and the two molecules show immunological partial cross-reactivity (see below).

### Further characterization of sialoprotein II

Sialoprotein II, which was the predominating extractable sialoprotein from bovine bone, was further analysed. Its molecular mass was 57300 Da determined by sedimentation-equilibrium centrifugation in 6 M-guanidinium chloride (Fig. 5a). The sedimentation coefficient ( $s_{20,w}^0$ ) was found to be 2.53 S (Fig. 5b). The determination of molecular mass presented some problems, since sialoprotein II showed a concentration-dependent aggregation even in the presence of 6 M-guanidinium chloride. This aggregation was, however, concentration-dependent, and not observed at initial concentrations of less than 0.380 mg/ml. The molecular mass was therefore calculated from the three lower sample concentrations (Fig. 5a). No aggregation was observed on sedimentation-velocity centrifugation even at high sample concentrations, and sialoprotein II sedimented as one symmetrical peak throughout the entire length of the centrifuge cell. Therefore it is possible that the aggregation is also pressure-dependent. The molecular mass and sedimentation coefficient were calculated by using a value for the partial specific volume of 0.66 ml/g, determined in 6 M-guanidinium chloride, by the method of Kratky *et al.* (1973). Interestingly, the sedimentation coefficient for sialoprotein II was lower than expected from its molecular mass. Such a discrepancy can, however, result from a high frictional ratio, i.e. the molecule being very asymmetric. Further information was obtained on electron microscopy of sialoprotein II with the use of shadow casting. The sialoprotein appeared as an extended rod with an average length of the core protein of 40 nm (Fig. 6), quite consistent with the high frictional ratio observed in the analytical centrifugations.

The sialic acid-containing oligosaccharides were isolated and analysed. After fragmentation of the core protein by alkaline-borohydride treatment, two differently sized oligosaccharides containing sialic acid could be separated on a Bio-Gel P-10 column (Fig. 7). The elution positions for oligomers of hyaluronic acid (HA) of known size (prepared by the procedure of Hascall & Heinegård, 1974) were determined for reference. About 25% of the sialic acid-containing oligosaccharides from sialoprotein II were eluted in a broad peak, corresponding to an average elution position of seven hyaluronic acid disaccharide units (HA14). The remaining material was eluted in a sharp peak corresponding to the elution





**Fig. 6. Electron microscopy of sialoprotein II**

A solution of sialoprotein II in 0.2 M-NH<sub>4</sub>HCO<sub>3</sub> was sprayed on to a mica grid and shadowed with platinum/carbon (Kühn *et al.*, 1981). Magnification  $\times 112\,500$ . The bar indicates 100 nm. (Picture by courtesy of Ms. H. Wiedeman and Dr. R. Timpl, Max-Planck-Institut für Biochemie, Martinsried, West Germany.)

position of an oligomer of three hyaluronic acid disaccharide units (HA6). The chromatogram was pooled to give the two fractions indicated in Fig. 7. The oligosaccharides in the leading peak (fraction I) contained mannose, galactose and glucosamine. Their content of galactosaminitol was negligible, showing that they were not linked by an *O*-glycosidic linkage to the protein. It is therefore likely that these oligosaccharides are attached to the core protein via an alkali-resistant *N*-glycosidic linkage (Table 2). The oligosaccharide fraction II, which was eluted retarded on the column, contained mannose, galactose and galactosaminitol (Table 2), indicating that these oligosaccharides represent *O*-glycosidically linked oligosaccharides. It was somewhat surprising, however, to find mannose residues in these oligosaccharides, since mannose is a residue primarily associated with *N*-glycosidically linked oligosaccharides. Fraction II could, however, contain some small *N*-glycosidically linked oligosaccharides. Whether there are two different types of oligosaccharides in fraction II or mannose and galactosaminitol are both residues of an unidentified type of oligosaccharide remains to be determined. In summary, sialoprotein II seems to have the shape of an extended rod containing a core protein with a very high content of aspartic acid/asparagine and glutamic acid/glutamine. Many of the serine and threonine residues appear to be substituted with a large number of negatively charged oligosaccharides of short length.

### Immunochemical analysis of sialoprotein II

Antibodies directed to sialoprotein II were raised in rabbits, and an enzyme-linked immunosorbent assay was developed. Upon inhibition assay, the lower limit for quantification of sialoprotein II was about 15 ng/ml (Fig. 8a). The assay was used to determine remaining antigenicity of sialoprotein II after chemical and enzymic treatments (Fig. 8a), mainly to study the nature of the antigenic determinants in the molecule. The antigenicity was almost unaffected by reduction and alkylation, indicating that no disulphide bond was essential for the antigenicity. Interestingly, some 50% of the antigenicity of sialoprotein II remained after digestion with trypsin, whereas the antigenicity of sialoprotein II was completely lost after alkaline-borohydride treatment, indicating that the antibodies were not directed against the oligosaccharide side chains. Inhibition assays also revealed that sialoprotein II showed only partial cross-reactivity with sialoprotein I (Fig. 8b). Interestingly, the inhibition curve produced by sialoprotein I was quite similar to that produced by trypsin digests of sialoprotein II, indicating that the common antigenic determinants were resistant to the enzyme treatment. The enzyme-linked immunosorbent assay was also used to determine the distribution of sialoprotein II among extracts of bovine tissues other than bone, i.e. aorta, cartilage, dentine, kidney, liver, muscle, sclera, skin and tendon. Some extracts contained components that, even after dilution, were precipitated,



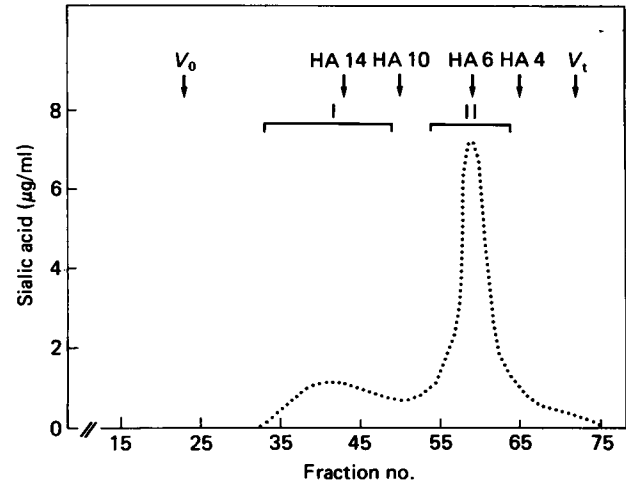


Fig. 7. Bio-Gel P-10 chromatography of oligosaccharides from sialoprotein II

Oligosaccharides were liberated from the core protein of sialoprotein II by alkaline-borohydride treatment. The alkaline-borohydride digest was chromatographed on a Bio-Gel P-10 column eluted at 20 °C with 1 M-pyridine/acetate buffer, pH 7, at a flow rate of 2 ml/h. The fractions were freeze-dried (to remove pyridine/acetate), dissolved in distilled water and analysed for content of sialic acid (µg/ml). The void volume ( $V_0$ ) and total volume ( $V_t$ ) were determined by chromatography of intact sialoprotein and sialic acid respectively. Elution positions for hyaluronic acid oligomers (HA) of known size were used as reference. The chromatogram was pooled to give two fractions (I and II) as indicated in the Figure.

Table 2. Composition analysis of sialoprotein II oligosaccharides isolated by Bio-Gel P-10 chromatography (Fig. 7)

Sugar	Composition of fraction I (residues/glucosamine residue)	Composition of fraction II (residues/galactosamine residue)
Sialic acid	1.24	7.70
Galactosamine*	0.02	1.00
Glucosamine	1.00	0.08
Galactose	0.62	0.93
Mannose	0.30	1.86

\* Determined as galactosaminitol.

making accurate enzyme-linked immunosorbent assay impossible. Therefore, before enzyme-linked immunosorbent assay we purified the sialoproteins by DEAE-cellulose ion-exchange chromatography (for details see the Materials and methods section). To test the fractionation technique, a known amount of sialoprotein fraction (DE51 III, Fig. 1) was dissolved in the extraction medium (see the Materials and methods section) and taken through the same purification sequence. All sialoprotein antigenicity was obtained in the fraction eluted with 0.7 M-sodium acetate, and about 95% of the sialoprotein was recovered. Thus, when purified fractions from the various extracts were analysed, only bone contained material that reacted in the assay and was eluted at 0.7 M-sodium acetate (detailed results not shown). Since no inhibition was shown by samples of

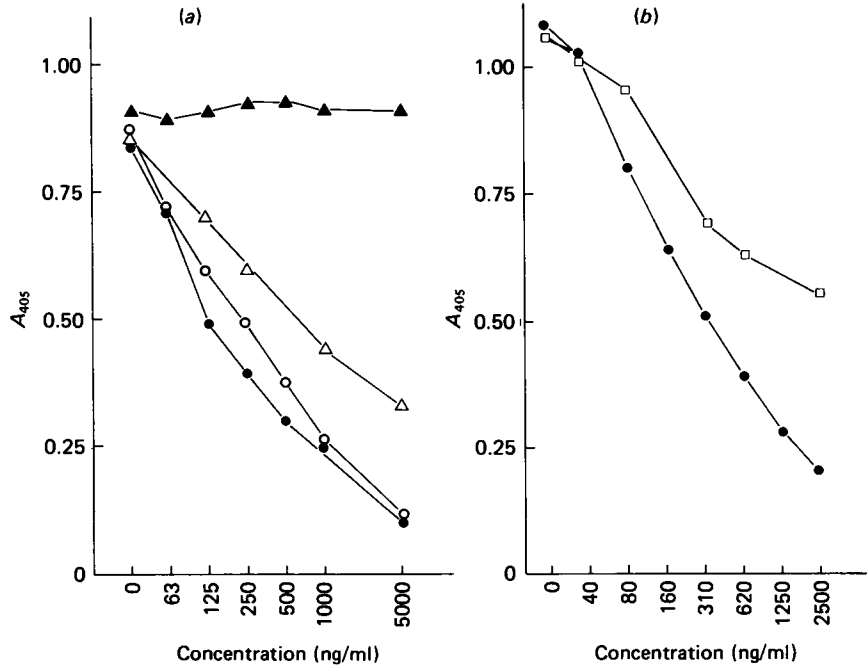


Fig. 8. Enzyme-linked immunosorbent assay of sialoprotein II

(a) Fractions of intact sialoprotein II (●) as well as of sialoprotein II treated with alkaline borohydride (▲), treated with trypsin (△) or reduced and alkylated (○) were tested in an inhibition assay. (b) Cross-reactivity between sialoprotein I (□) and II (●) analysed by using an enzyme-linked immunosorbent assay for sialoprotein II. Experimental details are given in the Materials and methods section.

other bovine tissue extracts, the sialoproteins represent molecules unique to the osteogenic phenotype. The extractable fraction of the sialoprotein in bone was calculated to be about 1.5 mg/g wet wt. of bone, compared with the detection level for sialoprotein II of about 100 ng/g wet wt. of tissue.

This work was supported by grant from the Swedish Medical Research Council (05668), Folksam's Yrkesskadors Stiftelse, Kocks Stiftelser, Österlunds Stiftelse, Konung Gustaf V:a 80-års fond and the Faculty of Medicine, University of Lund. Skilful technical assistance was provided by Ms. Sara Axelsson and Ms. Britt Mattsson. Advice and help in determination of the partial specific volume of sialoprotein II by Dr. Ingemar Carlstedt is appreciated. We gratefully acknowledge the work of Ms. Hanna Wiedeman and Dr. Rupert Timpl at the Max-Planck Institut für Biochemie, Martinsried, West Germany, in preparing the electron-microscopical pictures of sialoprotein II by using rotary-shadowing techniques.

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Received 22 April 1985/11 July 1985; accepted 27 August 1985