Carbohydrate Research, 71 (1979) 273–285

ISOLATION AND CHARACTERIZATION OF GLYCOSAMINOGLYCANS (MUCOPOLYSACCHARIDES) FROM THE SKIN OF THE FISH *Labeo rohita**

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(Received August 28th, 1978, accepted for publication, September 13th, 1978)

ABSTRACT

Hot-water extraction of defatted skin of the fish *Labeo rohita* yielded a viscous, glycoprotein solution This was extensively digested with pronase, and then treated with trichloroacetic acid to remove the proteins and nucleic acids On precipitation with ethanol, the solution furnished a mixture of several glycosaminoglycans which was fractionated by complexing with cetylpyridinium chloride and alkaline copper solution to yield three pure fractions From analyses, specific rotation values, i r data, and enzymic studies, the three fractions were fully characterized to be dermatan sulfate, chondroitin 4-sulfate, and hyaluronic acid The viscosity-average molecular weight of dermatan sulfate was found to be $2\,3 \times 10^4$, and that of hyaluronic acid, $1\,78 \times 10^5$.

INTRODUCTION

The mucopolysaccharides of various mammalian skins have been investigated[1–6] extensively, but very few reports[7,8] are available on similar biopolymers isolated from aquatic sources This paper deals with the isolation and characterization of three glycosaminoglycans, *viz*, dermatan sulfate (DS), chondroitin 4-sulfate (CS-A), and hyaluronic acid (HA) from the skin of the fish *Labeo rohita*

Labeo rohita is a very common, Indian fish available throughout the year, and it serves as one of the major sources of protein in India In appearance and taste, it closely resembles the American carp (*Cyprinus carpio*)

EXPERIMENTAL

Materials and methods — Standard hyaluronic acid (from human umbilical cords), bovine serum albumin, and ovine testicular hyaluronidase (specific activity 1880 units/mg) were obtained from Biochemicals Unit, V P Chest Institute, New Delhi Pronase was obtained as a gift from Cal Biochem, U S A (45,000 PUK/g)

*Part I of a series
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and from E Merck, Germany (70,000 PUK/g) L-Iduronic acid (as the calcium salt) was kindly provided by Dr J A Cifonelli, University of Chicago, Illinois, U S A

The skins were collected from live *Labeo rohita* fishes (weighing 0 5 to 1 0 kg), after removal of the scales, the skins were defatted by extraction with several changes of cold acetone, and as much as possible of the extraneous tissues was scraped out The cleaned skins were then cut into small pieces, air-dried, and kept frozen

Paper partition-chromatography (hereafter, "chromatography") was conducted on Whatman No 1 paper by the descending technique, using the following solvent-systems (v/v), (*A*) 8 2 1 ethyl acetate–pyridine–water, (*B*) 5 5 1 3 ethyl acetate–pyridine–acetic acid–water, (*C*) 10 1 2 1-butanol–ethanol–water, (*D*) 4 1 1 1-butanol–ethanol–water[9], and (*E*) 8 2 3 *tert*-amyl alcohol–formic acid–water[10] The staining agents used were (*a*) alkaline silver nitrate[11], and (*b*) a 0 5% solution of ninhydrin in acetone

Electrophoresis[12] of polysaccharides was performed on strips (5 × 10 cm) of cellulose acetate in 0 15M formic acid–pyridine buffer, pH 3 0, at a current strength of 2 5–3 0 mA at 17 V/cm, for 30 min at room temperature (~25°) The strips were then stained with Alcian Blue[13]

All specific rotations were recorded at equilibrium with a Perkin–Elmer Spectropolarimeter, model 241 at 20° Spectrophotometric determinations were performed with a Pye–Unicam (model SP500) and a Beckman (model IR-20-A) spectrophotometer For i r spectra, the respective polysaccharide sample (~1 mg) was incorporated into a cesium bromide pellet and compared with air as the blank. High-speed centrifugation was conducted with a Beckman ultracentrifuge, model L5-65 Viscosity measurements were made with a Cannon–Fenske viscometer, model F/NO 50/99 G l c. experiments were conducted in a Hewlett–Packard Gas Chromatograph, model 5730A, equipped with a flame-ionization detector, nitrogen being used as the carrier gas. Glass columns (1 83 m × 6 mm) were used, respectively containing (*I*) 3% of ECNSS-M supported on Gas Chrom Q (100–120 mesh), and (*II*) 3% of Poly A-103 on Gas Chrom Q (100–120 mesh)

Unless otherwise stated, all evaporations were performed at 35–40° under diminished pressure De-ionization was effected with Dowex 50W-X8 (H^+) (20–50 mesh) and Dowex 1-X4 (formate or hydrogencarbonate form) (20–50 mesh) ion-exchange resins All pure preparations were isolated in the form of sodium salts

Identification of components (monosaccharides and amino acids) — Different conditions of hydrolysis (in sealed tubes) were used for the identification of neutral, acidic, and amino sugars and for amino acids

(*a*) *Neutral sugars* — The respective polysaccharide (~2 mg) was hydrolyzed[14] (M HCl, 1 mL, 3 h, 100°) The hydrolyzate was diluted with water (20 mL) and passed through columns of Dowex 1-X4 (HCO_3^-) and Dowex 50 (H^+) resins The effluents and washings were concentrated, and examined by chromatography (solvents *A* and *C*, staining agent *a*) The rest of the hydrolyzate was reduced with potassium borohydride, and the alditols converted[15] into their peracetates for g l c (Col *I*, 195°)

(*b*) *Hexuronic acids* — The polysaccharide sample (~1 mg) was hydrolyzed

(M HCl, 1 mL, 1 h, 100°) The hydrolyzate was concentrated, and hydrochloric acid was completely removed by repeated addition and evaporation of water Hexosamines were adsorbed on a column (0 8 × 4 cm) of Dowex 50 (H^+) resin, and the effluents containing the acidic components were concentrated and subjected to chromatography[10] (solvent *E*, staining agent *a*)

For g l c , the uronic acids (40–50 μg) in a portion of the foregoing hydrolyzate were converted into the corresponding alditol acetates (*via* the aldonic lactone, and reduction) as described by Lindahl *et al*[16] Alditol acetates of standard D-glucuronic and L-iduronic acids were prepared similarly.

(*c*) *Hexosamines* — The respective polysaccharide (~2 mg) was hydrolyzed (6M HCl, 2 mL, 6 h, 100°) After complete removal of hydrochloric acid, the hydrolyzate was examined by chromatography (solvent *B*, staining agents *a* and *b*) A portion of this hydrolyzate was subjected to ninhydrin degradation by a modification[9] of the method of Stoffyn and Jeanloz, and the resulting pentoses were identified by chromatography, and by g l c of their alditol acetates Hexosamines were also identified by g l c (Col *II*, 200°) of their alditol acetates as described by Neidermeier *et al*[17]

(*d*) *Amino acids* — The polysaccharide sample (~1 mg) was hydrolyzed (6M HCl, 1 mL, 8 h under N_2, 100°) The hydrolyzate was concentrated and chromatographed (solvent *A*, staining agent *b*)

Estimations — Hexuronic acids were estimated by the carbazole[18] and Bitter-Muir[19] methods, using D-glucuronic acid as the standard

Hexosamines were estimated by g l c as described by Neidermeier *et al*[17] Samples containing suitable standards (*e g* , 2-acetamido-2-deoxy-D-galactose for estimating 2-amino-2-deoxy-D-glucose, and *vice versa*) were hydrolyzed as mentioned earlier Total hexosamines were determined by the Elson–Morgan procedure[20] after similar hydrolysis of the samples

Neutral sugars were estimated by g l c of their alditol acetates after similar hydrolysis (see previous section), using *myo*-inositol as the internal standard

The protein content of samples was measured by the method of Lowry *et al*[21], with bovine serum albumin as the standard

The sulfate content was determined by the procedure of Antonopoulos[22], using potassium sulfate as the standard

The carbazole orcinol (C/O)[23] ratio was determined by the carbazole[18] and orcinol[24] methods, using a heating time of 20 min, and the proportion of iduronic acid and of glucuronic acid was assessed[25] from this ratio

The effect of testicular hyaluronidase on the glycosaminoglycan preparations was studied turbidimetrically[26]

Isolation of the crude glycosaminoglycans — Defatted, dry skin (28 g) was suspended in water (500 mL), and homogenized in a Waring Blendor for 15 min (with intermissions) The suspension was heated on a boiling-water bath for 1 h, with stirring, and filtered hot through Nylon cloth The filtrate was centrifuged (6000 r p m , 1 h, 25°), and the product in the clear, supernatant liquor was precipi-

tated as a gummy material with ethanol (95%, 3 vol) The precipitate was collected at the centrifuge, and dried with acetone

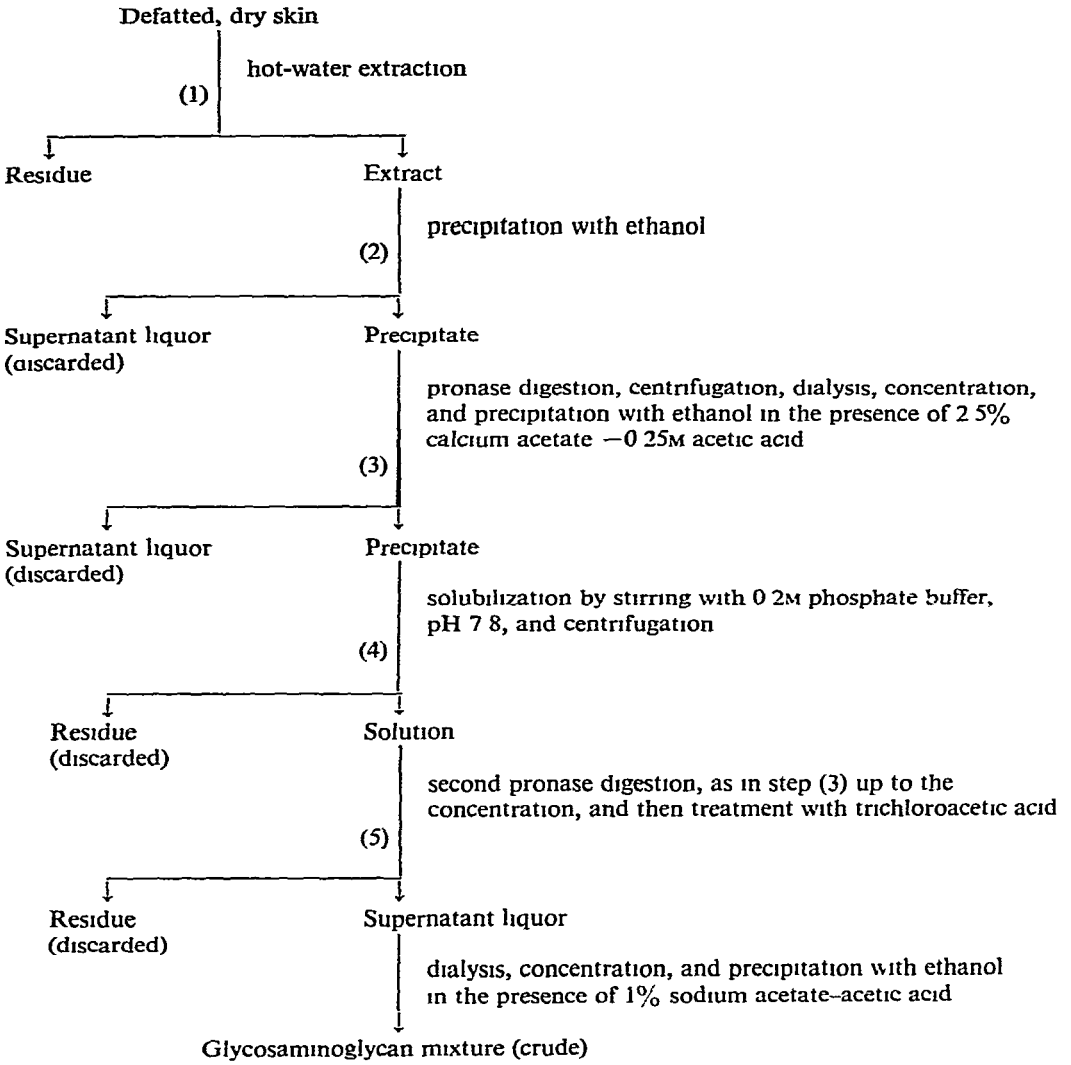
The dry precipitate* (18 g) was dissolved in 0 2M sodium phosphate buffer, pH 7.8, containing 1 5mM calcium chloride, and digested with pronase for 96–120 h at 37°. The substrate concentration was maintained at 25 mg/mL of buffer, and the enzyme was added, as 1% of the substrate, in two instalments A few drops of toluene were added, to prevent bacterial growth After centrifugation and extensive dialysis at 4°, the digested solution was concentrated (to ~100 mL) and its contents precipitated with ethanol (95%, 2 vol) in the presence of 2 5% calcium acetate–0 25M acetic acid

The precipitate was mixed with the same buffer, and the suspension was centrifuged. The residue was discarded, and the solution was dialyzed against the same buffer (to remove traces of ethanol) This material was redigested (48 h) with pronase, and the suspension centrifuged The supernatant liquor was dialyzed, and concentrated (~60 mL); to the concentrate in an ice bath was slowly added aqueous trichloroacetic acid (40%, 20 mL) with stirring and the mixture was kept for 30 min The precipitate was centrifuged off, and the supernatant liquor was dialyzed against distilled water at 4° The dialyzed solution was further clarified by centrifugation (19,000 r p m , 1 h, 5°), and concentrated to ~15 mL The glycosaminoglycans were precipitated with ethanol (95%, 4 vol) in the presence of 1% sodium acetate–acetic acid (pH ~4 0). The precipitate was collected by centrifugation, and dried by successive trituration with 95% ethanol, absolute ethanol, and dry ether, yield 75 8 mg The process of isolation is summarized in Scheme 1

Fractionation[26] *of the crude glycosaminoglycans by cetylpyridinium chloride* (*CPC*) — The crude preparations (200 mg) from three different lots were mixed with 0 3M sodium chloride solution (50 mL), and kept for 15 min at 35°, and then aqueous CPC solution (2%, 3.5 mL) was added dropwise with stirring The mixture was kept overnight at room temperature, and the precipitate was collected at the centrifuge, washed with 0 3M sodium chloride solution (3 × 5 mL), and dissolved in 2M sodium chloride solution (10 mL) containing a few drops of methanol The material was reprecipitated with 75% (v/v) ethanol (25 vol) The precipitate (fraction A) was collected by centrifugation, and dried *in vacuo* over P_2O_5, yield 34 5 mg

During dialysis of the supernatant liquor from the precipitation with CPC, a gummy residue (fraction B) gradually accumulated in the bag This was saved, and the supernatant liquor was concentrated (~30 mL) and made 0 03M with respect to sodium chloride. To this was added CPC solution (2%, 4 mL) as described earlier, whereupon a gummy aggregate (fraction B) appeared Both B-fractions were separately washed with 0 03M sodium chloride solution (3 × 5 mL), and then pooled by solubilizing with 1 .1 (v/v) 2M sodium chloride–methanol (15 mL) The solution was

*In subsequent lots, isolation of this solid was avoided; instead, the requisite amounts of solid NaH_2PO_4, Na_2HPO_4, and $CaCl_2$ were directly added to the water extract, the pH was adjusted, and the material was digested with pronase

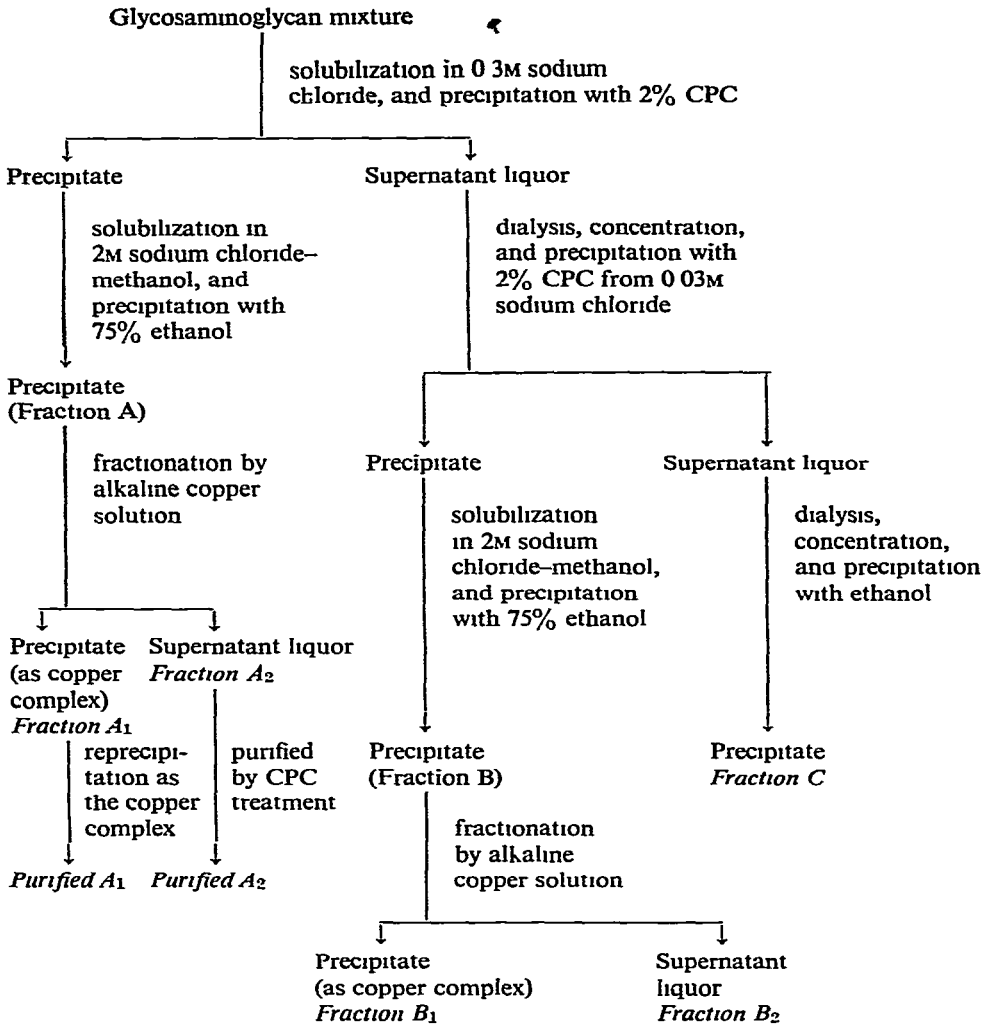


Scheme 1 Isolation of the crude mixture of glycosaminoglycans from the skin of the fish *Labeo rohita*

clarified by centrifugation, and the contents precipitated with ethanol (75%, 10 vol) The precipitate (fraction B) was collected in the usual way, yield 32 8 mg

After dialysis, concentration, and ethanol precipitation, the supernatant liquor from fraction B yielded a greenish precipitate (fraction C), yield 30 3 mg

Further fractionation and purification of fractions A and B by complexing with alkaline copper solution[27] — On treatment with alkaline copper solution in a nitrogen atmosphere, fraction A (100 mg) was separated into fractions A_1 (40 mg, from the precipitate) and A_2 (50 mg, from the supernatant liquor of A_1) Similarly, fraction B (100 mg) yielded fractions B_1 (60 mg, from the precipitate) and B_2 (12 mg, from the supernatant liquor) Fraction A_1 (35 2 mg) was once again purified through the



Scheme 2 Fractionation of the crude glycosaminoglycan preparation to obtain the pure components (A_1, A_2, and B_1).

alkaline copper complex, to yield purified A_1 (30 5 mg) Fraction A_2 (45 mg) was purified by repeating the CPC treatment from 0 3M sodium chloride solution, to yield purified A_2 (36.8 mg) The fractionation procedures employing CPC and alkaline copper solution are summarized in Scheme 2

RESULTS AND DISCUSSION

Isolation — Hot-water extraction of defatted skins furnished a viscous solution that was processed to afford a solid By hydrolysis, this solid was found to be essentially a glycoprotein In order to isolate the carbohydrate components, the glycoprotein

TABLE I

IDENTIFICATION OF THE MONOSACCHARIDES IN THE HYDROLYZATES OF THE CRUDE GLYCOSAMINOGLYCAN

Sugars detected	R_{Glc}[a]			R_{GlcA}[b]	$R_{\Delta D}$[c]	*RRT*[d]	
	A[e]	*B*[e]	*C*[e]	*E*[e]	*D*[e]	*Col I, 195°*	*Col II, 200°*
Glucuronic acid	—	—	—	1 00	—	1 00	—
Iduronic acid	—	—	—	1 32	—	0 50	—
Glucuronolactone	—	—	—	1 68	—	—	—
Iduronolactone	—	—	—	2 11	—	—	—
2-Amino-2 deoxyglucose	—	0 88	—	—	1 00 (Ara)	0 31 (arabinitol pentaacetate)	1 00
2-Amino-2 deoxygalactose	—	0 82	—	—	1 20 (Lyx)	0 29 (lyxitol pentaacetate)	1 18
Galactose	0 90	—	0 84	—	—	0 88	—
Glucose	1 00	—	1 00	—	—	1 00	—
Mannose	—	—	—	—	—	0 77	—
Fucose	1 33	—	1 52	—	—	0 20	—

[a] R_{Glc} values refer to the chromatographic mobility relative to that of D-glucose [b] R_{GlcA} values refer to the chromatographic mobility relative to that of D-glucuronic acid [c] $R_{\Delta D}$ values refer to the chromatographic mobility of the pentoses obtained by ninhydrin degradation of the hexosamines, relative to that of D-arabinose [d] RRT values refer to the retention times relative to that of D-glucitol hexaacetate [e] A, B, C, D, and E refer to chromatography solvents (see text)

(18 g) was extensively digested with pronase, and the mixture dialyzed The carbohydrate components, together with some protein, remained within the bag, this showed that the carbohydrate components were not mono- or oligo-saccharides The product of the first pronase digestion was redigested with the same enzyme, and, after dialysis and concentration, the solution was treated with trichloroacetic acid to remove the remaining protein and nucleic acids The excess of trichloroacetic acid was removed by extensive dialysis, and the solution was clarified by centrifugation (19,000 r p m) The supernatant liquor was concentrated, and the contents precipitated with ethanol in the presence of sodium acetate–acetic acid (pH ~4 0) to yield the crude glycosaminoglycan (75 8 mg)

By chromatography and g l c , the hydrolyzate of this crude mixture was found to contain glucuronic acid and iduronic acid (and their lactones), 2-amino-2-deoxy-galactose and -glucose, fucose, galactose, glucose, and mannose (see Table I)

The polysaccharide preparation was, therefore, a mixture of several glycosaminoglycans It is to be noted that xylose (detected later, by g l c only) could not be found at this stage

Fractionation by CPC — Fractionation of the foregoing polysaccharide mixture (200 mg) was effected by precipitation with CPC from aqueous sodium chloride solutions of differing molarity, essentially according to the method described by Mathews[26] Precipitation from 0 3M sodium chloride solution furnished fraction A

TABLE II

COMPOSITION AND VALUES OF THE SPECIFIC ROTATION OF POLYSACCHARIDE FRACTIONS[a]

Fraction	$[\alpha]_{589.5}$ *(degrees)*[b]	*Sugars*[c]	*Relative proportions*[d]	*Amino acids*[e]
A (Chondroitin sulfates)	−56.0	glucuronic acid	+++	+
		iduronic acid	+++	
		2-amino-2-deoxygalactose	+++	
		2-amino-2-deoxyglucose	+	
		galactose	++	
		glucose	±+	
		mannose	+	
		fucose	+	
B (Hyaluronic acid)	−23.1	glucuronic acid	+++	+
		iduronic acid	±	
		2-amino-2-deoxygalactose	∓	
		2-amino-2-deoxyglucose	±++	
		galactose	+	
		glucose	++	
		mannose	+	
		fucose	+	
C	+29.7	hexuronic acid	nil	++
		2-amino-2-deoxygalactose	+	
		2-amino-2-deoxyglucose	+	
		galactose	++	
		glucose	+−+	
		mannose	±	
		fucose	++	

[a]Obtained by cetylpyridinium chloride fractionation of the crude mucopolysaccharides [b]In water [c]Detected by chromatography and g l c [d]±+++, major, ++, medium, and +, trace [e]One or more, as detected by chromatography (solvent *A*, staining reagent *b*)

(34.5 mg), having $[\alpha]_{589.5}$ −56.0° (*c* 1.7, water), whose major monosaccharide components were glucuronic acid, iduronic acid, and 2-amino-2-deoxygalactose, the other sugars were present in smaller proportions or traces (see Table II) The supernatant liquor from fraction A was adjusted to 0.03M with respect to sodium chloride, and the contents precipitated with CPC to yield fraction B (32.8 mg), $[\alpha]_{589.5}$ −23.1° (*c* 0.95, water) Fraction B was found to contain mainly glucuronic acid and 2-amino-2-deoxyglucose in the ratio of ~1:1, other components (as in fraction A) were present in traces (see Table II)

Dialysis, concentration, and ethanol precipitation of the supernatant liquor from fraction B finally yielded a greenish precipitate (fraction C, 30.3 mg), this had $[\alpha]_{589.5}$ +29.7° (*c* 0.8, water) Its constituents were mainly the neutral sugars and some amino acids Only traces of 2-amino-2-deoxy-galactose and -glucose could be detected in it, and there was no uronic acid (see Table II) However, due to its very heterogeneous character, fraction C was not further investigated

From these results, it was apparent that fraction A (whose major constituents

were glucuronic acid, iduronic acid, and 2-amino-2-deoxygalactose) was a mixture of chondroitin sulfates, and fraction B (having glucuronic acid and 2-amino-2-deoxyglucose as the major constituents) was presumably hyaluronic acid The preparations were not, however, sufficiently pure at this stage for a final decision

Further fractionation and purification of fractions A and B — Both fractions A and B were further fractionated by precipitation with alkaline copper solution essentially according to the method of Cifonelli *et al*[27] Fraction A (containing the chondroitin sulfates) furnished fraction A_1 (later characterized as being dermatan sulfate), and fraction A_2 (characterized later as being chondroitin 4-sulfate) A_1 (containing mainly iduronic acid and 2-amino-2-deoxygalactose) had $[\alpha]_{589.5}$ −65 5°, and a xylose galactose (molar) ratio of 1 5 On repeating the copper-complex treatment, the value of the specific rotation changed to −67 4°, and the molar ratio of xylose galactose became 1 2 (determined by g l c) Similarly, when A_2 was purified by repeating the CPC treatment, its specific rotation value changed from −18 0° to −28 0°, and the xylose . galactose ratio from 1 3 to 1 2 (from g l c) Purified A_1 and A_2 were completely free from fucose and mannose (see Tables II and III) However, the other analytical values did not show any significant variation, and, also, practically no further change in the composition could be achieved by repeating the purification processes

On purification (as the copper complex), fraction B yielded fraction B_1 (later characterized as being hyaluronic acid) and fraction B_2 This treatment freed B_1 of fucose and mannose, and its specific rotation value (−23.1° for B, see Table II) became −69 0° B_2 had $[\alpha]_{589.5}$ +10 7° (*c* 1 3, water) and contained mainly the

TABLE III

COMPOSITION AND CHARACTERISTICS OF PURIFIED GLYCOSAMINOGLYCAN PREPARATIONS A_1, A_2, AND B_1

Polysaccharide fraction	$[\alpha]_{589.5}$ *(degrees)*[a]	*Composition*				*Electrophoretic mobility* (R_{HA})[b]	*Neutral sugars*	*Amino acid*[c]
		Hexuronic acid (%)	*Hexosamine (%)*	*Sulfate (%)*	*Protein (%)*			
A_1 (DS)	−67 4 (0 76)	17 5[d] 23 0[e]	22 7	15 3	1 8	2 0	xylose galactose = 1 2, glucose, 0 2%	serine
A_2 (CS-A)	−28 0 (2 4)	21 8[d]	24 3	16 0	2 0	2 8	xylose galactose = 1 2, glucose, 0 6%	serine
B_1 (HA)	−69 0 (0 77)	44 4[d]	36 2	—	0 8	1 0	glucose, 0 6%, galactose, 0 17%	—

[a]In water, concentration in parentheses [b]R_{HA} refers to relative electrophoretic mobility with respect to standard hyaluronic acid [c]Identified by comparative chromatography (solvent *A*, staining reagent *b*) with standard DL-serine [d]Carbazole [e]Bitter–Muir

neutral sugars, along with traces of uronic acids and hexosamines. The compositions of purified A_1, A_2, and B_1 are given in Table III

Characterization of purified fraction A_1 (dermatan sulfate, DS) — Purified A_1, having $[\alpha]_{589.5}$ −67.4° (lit.[28] −60.0 to −70.0°, for DS), contained hexuronic acids 17.5% (carbazole*), 23% (Bitter–Muir) [lit.[30] 12.9–19.3% (carbazole)], galactosamine 22.7% (lit.[30] 21.9–27.8%); sulfate 15.3% (lit.[12] 15.6%), protein 1.8% (lit.[14] 1.7%), and molar ratio of xylose galactose 1.2 (lit.[14] Xyl·Gal = 1.2) These results are given in Table III The material had C/O, 0.41 (lit.[31] 0.41), and iduronic acid glucuronic acid ratio, 9.1 (lit.[25,31] 9.1), it was electrophoretically homogeneous, having a mobility[12] twice that of hyaluronic acid (see Fig 1)

The i.r. spectrum of this fraction showed absorption bands at 1260–1230, 928, 840, and 712 cm^{-1}, characteristic[28,32] of dermatan sulfate (DS) The band at 1260–1230 cm^{-1} confirms the presence[33] of sulfate groups From these results, purified A_1 was conclusively identified as being dermatan sulfate

The viscosity-average molecular weight ($M\nu$) of this DS was determined by using the Mathews equation[34], $\eta_{sp}/C = [\eta] = 3.1 \times 10^{-4} \times M\nu^{0.74}$, and the value was found to be 2.3×10^4, this is in the range of $M\nu$ reported[30] for pig-skin DS In the foregoing equation, the reduced viscosity (η_{sp}/C) was determined at 30°, at a concentration of 2 mg of polysaccharide/mL of 0.15M sodium chloride–0.1M acetate buffer, pH 5.0, and the value was found to be 0.524 dL g^{-1} At this concentration, the reduced viscosity has been reported[30] to be very close to the intrinsic viscosity

The presence of glucuronic acid (~10%) in our preparation of DS is consistent with the fact that dermatan sulfates have a copolymeric structure[12,30]

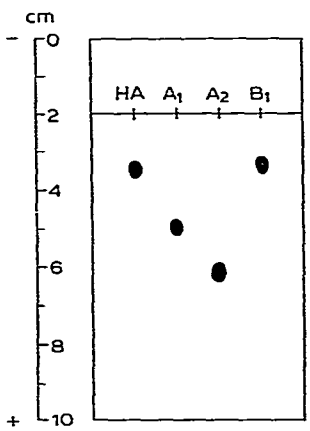


Fig 1 Electrophoresis of purified A_1 (DS), A_2 (CS-A), and B_1 (HA) on cellulose acetate strips in 0.15M formic acid–pyridine buffer, pH 3.0 (HA = Standard hyaluronic acid)

*It has been reported[29] that, in the carbazole method, iduronic acid and its derivatives give 25–35% of the color given by an equivalent weight of glucuronic acid

Characterization of purified fraction A_2 (chondroitin 4-sulfate, CS-A) — Purified A_2 had the composition glucuronic acid, 21 8% (carbazole), galactosamine, 24.3%, sulfate, 16 0%, protein, 2 0%, and xylose·galactose molar ratio, 1 2 (see Table III) It was electrophoretically homogeneous (mobility[12], 2 8 × that of hyaluronic acid, see Fig 1) These values are comparable to those in earlier reports[35,37] for chondroitin sulfates, and the value of the specific rotation, namely, −28 0° (lit [28] −28 0 to −33 0°, for CS-A) indicated it to be CS-A The final identification of this fraction as CS-A was established from its i r spectrum, which showed absorption bands at 1260–1230, 928, 882, 850, and 725 cm^{-1} As reported[28 32] earlier, these bands, especially that at 850 cm^{-1}, decisively establish the position of the sulfate groups at C-4 of the hexosamine residues The identification was fully corroborated by the results of a turbidimetric study[26] (disappearance of turbidity at a lower rate[36]) with testicular hyaluronidase

The presence of xylose and galactose (in the molar ratio of 1 2), and of serine, in our DS and CS-A preparations is consistent with previous reports[14 37 38] in which they have been shown to be present at the carbohydrate–protein linkage through serine

Characterization of fraction B_1 (hyaluronic acid, HA) — Fraction B_1 had the following composition (see Table III) glucuronic acid, 44 4% (lit [39] 44 4%), 2-amino-2-deoxyglucose, 36 2% (lit [39] 35 1%), and protein, 0 8% These results, and the value of the specific rotation, namely, −69 0° (lit [28 29] −70 0 to −80 0°, for HA), indicate that this fraction is hyaluronic acid This conclusion was corroborated by its electrophoretic mobility[12 39] (see Fig 1) compared with that of standard HA, and by the disappearance of turbidity with acid albumin[26] after incubation of this fraction with ovine testicular hyaluronidase Furthermore, the i r spectrum (4000–600 cm^{-1} region) of B_1 was completely superposable on that of reference HA This fraction is, therefore, identified as hyaluronic acid

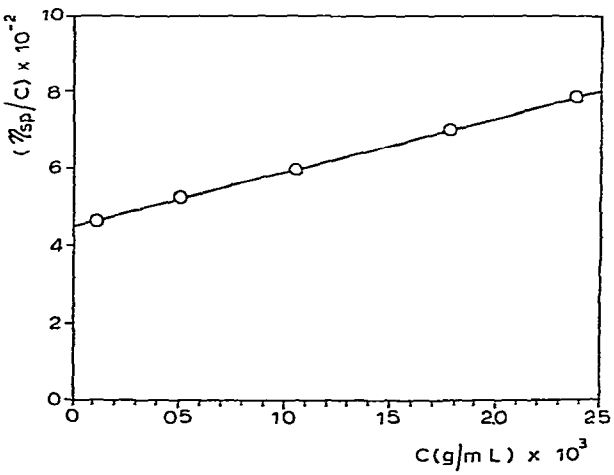


Fig 2 Plot of reduced viscosity (η_{sp}/C) *vs* concentration (C) for fraction B_1 (HA) (From the intercept, the intrinsic viscosity [η] was found to be 450 mL g^{-1})

Viscosity measurements for this HA were made over the concentration range of 0.12–2 37 mg/mL of 0 2M sodium chloride solution at 25° The intrinsic viscosity $[\eta]$ was then determined from the plot of η_{sp}/C *vs* C (see Fig 2), and was found to be 450 mL g^{-1}. The viscosity-average molecular weight ($M\nu$) of this polymer was then calculated by using the equation, $[\eta] = 3\,6 \times 10^{-2} \times M\nu^{0\,78}$, as described by Laurent *et al.*[40], and the value of $M\nu$ was found to be $1\,78 \times 10^5$, which is in the range given in earlier reports[28 40].

It may be noted that the presence of a small proportion of glucose in all three of the purified fractions (A_1, A_2, and B_1) is probably due to contamination with fraction C (whose major constituents are glucose and galactose, see Table II) Also, the presence of variable proportions of galactose (as evidenced by analysis during purifications) in all of the fractions clearly indicates that there was an additional source for it. However, the presence of neutral sugars other than galactose and xylose in some glycosaminoglycans from aquatic[8] and mammalian[41] sources has been reported

ACKNOWLEDGMENT

The authors are grateful to the U G C , New Delhi, for financial assistance and for a Fellowship to S K.S

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