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CHARACTERIZATION OF THE CHONDROITIN SULFATE PRODUCED BY B16 MOUSE MELANOMA CELLS*

VEERASINGHAM P. BHAVANANDAN AND EUGENE A. DAVIDSON

Department of Biological Chemistry, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033 (U. S. A.)

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ABSTRACT

The mucopolysaccharides produced by B16 mouse melanoma cells have been isolated in milligram quantities from the spent media in which the cells were grown in the presence of 2-amino-2-deoxy-D-glucose-*t* and [^{35}S]-sulfate. The mucopolysaccharides obtained by precipitation with cetylpyridinium chloride from the Pronase digest of the media were further purified by gel filtration, ion-exchange chromatography, and treatment with nucleases. The major components were identified as chondroitin-4-sulfates by identification of the hexosamine as 2-amino-2-deoxy-D-galactose, and by digestibility with hyaluronidases, chondroitinase AC, and chondro-4-sulfatase. The o.r.d. curve and i.r. spectra of these components also confirmed their similarity to chondroitin-4-sulfate from cartilage. The molecular weight of the polysaccharide chains was estimated to be in the range 90,000–120,000 by sedimentation equilibrium analysis.

INTRODUCTION

Mucopolysaccharides are associated with the surface of a wide variety of cultured normal and cancer cells[1,2]. They are thought to play a role in a variety of cell properties such as adhesion, contact inhibition, masking of receptors, and interaction with antigens. In addition, due to their high negative charge and their large size, they are also likely candidates for regulating macromolecule and cation access to the cell plasma membrane. This class of macromolecule is also associated with the nuclei[3–5], mitochondria[6], and plasma membrane[7]. Neither the nature of the association of mucopolysaccharides with these cell organelles nor their function is clearly understood.

A prior study of the complex saccharides produced by B16 mouse melanoma cells[8] showed the presence, both in the cells and spent media, of a family of chon-

*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

droitin sulfates having properties distinct from those of typical chondroitin sulfates of cartilage. This class of polymer was also the major mucopolysaccharide associated with the nucleus of these cells[3].

We now report on the chemical and physical characterization of the chondroitin sulfates produced by B16 mouse melanoma cells in culture.

EXPERIMENTAL

Materials. — Fetal calf serum and antibiotic solution were obtained from Flow Laboratories, Rockville, MD, and other components for culture media from Grand Island Biological Company, Grand Island, N.Y. Pronase CB was obtained from Calbiochem; controlled-pore glass beads (CPG 10-240) and glyceryl-CPG 240 were from Electronucleonics, Fairfield, NJ; Sepharose 4B-200 and diethylaminoethyl (DEAE) Sephadex were from Sigma; Chondroitinases ACII (EC 4.2.2.5) and ABC (EC 4.2.2.4) and chondro-4-sulfatase (EC 3.1.6.9) were from Miles Laboratories; testicular hyaluronidase (EC 3.2.1.35) (400 units per mg) was from Worthington, Inc.; bacterial hyaluronidase (EC 4.2.99.1) from Organon; and leech hyaluronidase (EC 3.2.1.36) from Biotrics, Arlington, MA. Heparitinase was isolated from an adapted *Flavobacterium heparinum* strain provided by Dr. A. Linker[9,10]. Bovine pancreas deoxyribonuclease DN-EP (EC 3.1.4.5) was from Sigma, and bovine pancreas ribonuclease (EC 2.7.7.16) from Boehringer.

Vitreous humor hyaluronic acid was obtained from Worthington; proteoglycan and chondroitin-4-sulfate were isolated from pig-rib cartilage; and reference heparan sulfate was a gift from Dr. A. Linker. Streptococcal hyaluronic acid was a gift from Dr. Karl Meyer.

Column chromatography. — Glyceryl CPG-240 or CPG 10-240 beads (80–120 mesh) treated with polyethylene glycol were packed in silicone-coated columns with constant vibration according to the instructions of the manufacturers. The columns were equilibrated with 0.5M KCl and eluted with the same solution; a pump was used to maintain a constant flow rate of 30 ml/h. Columns (0.9 × 70 cm) of Biogel P2 were equilibrated and eluted with 0.1M pyridine acetate (pH 5.0). Sepharose 4B and Sephadex G200 were packed in columns (2 × 60 cm) and equilibrated with 50 mM Tris-HCl buffer (pH 8.0).

DEAE Sephadex A-25-120 was swollen in M sodium acetate for 48 h, filtered off, and washed with 0.01M pyridine acetate buffer (pH 5.1) on a Buchner funnel until the filtrate gave a negative test for chloride. A suspension of the beads in the same buffer was packed in a column (13 × 1 cm).

Cellulose acetate electrophoresis was performed in a Beckman R-101 microzone electrophoresis cell with 0.2M calcium acetate (pH 7.0) at 5 mA for 3 h, or pyridine–formic acid buffer (0.1M in formic acid, pH 3.0) at 10 mA for 20 min. Staining with Alcian Blue (0.1% in 0.5% acetic acid) was used for detecting acidic components (mucopolysaccharides and mucin-type sialoglycoproteins).

Unless specified otherwise, all dialysis was performed at 4° in the presence of toluene and chloroform.

Hexosamine determinations on isotopically labeled components were carried out, after acid hydrolysis, by ion-exchange chromatography on an automated amino acid analyzer; the stream-splitting technique described earlier[8,11] was used. The samples were hydrolyzed with 6M HCl at 110° *in vacuo* for 24 h; on the basis of control experiments, a correction for the destruction of 28.5% of 2-amino-2-deoxy-glucose and 25.5% of 2-amino-2-deoxygalactose was applied. G.l.c. identification of neutral sugars was performed, following hydrolysis as described in the text, by the alditol acetate procedure[12]. A Packard gas–liquid chromatograph was used[13] with a glass column (6 ft × 0.125 in.) of 3% of ECNSS-M on Gas Chrom Q (100–120 mesh). Uronic acids were identified as the trimethylsilyl derivatives[14] [15% of Apiezon M on Gas Chrom CLZ (80–100 mesh)]. Marker polysaccharides run on gel columns were assayed by the orcinol reaction[15]. Liquid scintillation counting was performed on an Intertechnique Model SL36 spectrometer; usually, 1-ml aqueous samples were mixed with 10 ml of the counting liquid containing xylene and Triton X-114[16]. Efficiencies for ^{3}H and ^{35}S were ~16 and ~47%, respectively, with a crossover of ~13–14% of ^{35}S into the ^{3}H channel. The citrate buffer fractions (4 ml) from the amino acid analyzer were mixed with 10 ml of 3a40 counting fluid (Research Products International Corporation) and counted. The efficiency for tritium in this system was 15%, using the same settings for minimal crossover of ^{35}S as above. Radioactivity on cellulose acetate or paper strips was estimated by extracting cut pieces with 1 ml of water in counting vials. After shaking for at least 6 h on a reciprocating shaker, counting liquid was added, and the solution was mixed and counted.

Cell cultures. — B16 mouse melanoma cell lines (B16C2 and B16C3) and an amelanotic clone were grown in 16-oz prescription bottles and subcultured at confluency by suspension with 0.02% of EGTA [ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid] in calcium- and magnesium-free phosphate buffered saline. The cells were cultured in Eagle's medium[17] containing twice the recommended concentration of vitamins and supplemented with non-essential amino acids (each at 0.1mM), sodium pyruvate (mM), and 10% heat-inactivated (56° for 30 min) fetal-calf serum. Cultures were routinely tested for bacterial contamination by the use of thioglycolate and tryptose phosphate broth[18], and for yeast and mold contamination with Sabouraud's medium[19]. The spent media at each medium change, and at subculturing, were decanted, centrifuged to remove floating cells, and stored in the freezer. We are grateful to Mr. John Banks for help in cell culture and for collecting media used in these studies. Spent medium containing labeled complex saccharides was prepared by culturing the cells for 48 h prior to harvest in medium containing (per ml) 10 μCi of 2-amino-2-deoxy-D-glucose-*t* (New England Nuclear, 755 mCi per mmol) and 50 μCi of $Na_2{}^{35}SO_4$ (New England Nuclear, 755 mCi per mmol).

Treatment of media and of fetal-calf serum with Pronase and alkali. — Pooled, frozen, spent media (~5 l, MI) was thawed and dialyzed at 4° against 0.9% NaCl for 2 days, and then against water for 5 days. The dialyzed material was concentrated by

ultrafiltration and lyophilization to ~600 ml and combined with 100 ml of dialyzed, labeled media. Calcium acetate was added (to 0.01M Ca^{2+}) and the pH was adjusted to 7.8 with 10M NaOH. Pronase (100 mg) was stirred in, toluene (2 ml) added, and the solution incubated in a stoppered flask at 40° for 72 h with further additions of 100 mg of enzyme and toluene after 24 and 48 h; the pH was maintained at 7.8–8.0 during the incubation by the addition of NaOH. The digest was centrifuged, the residue discarded, and the supernatant solution dialyzed against 0.9% NaCl overnight, and then against deionized water for 4 days. The contents of the dialysis bag were adjusted to pH 12.5 and, after 18 h at 20–25°, dialyzed for 5 days against several changes of deionized water. A second batch of spent media MII (3 l) was worked up in an identical manner, except that the treatment at pH 12.5 for 18 h was omitted.

As a control, heat-inactivated fetal-calf serum (300 ml) was digested with Pronase as described above for the second batch of media, and then dialyzed against 0.9% NaCl followed by deionized water for a total of 5 days.

Precipitation of digested media and serum with cetylpyridinium chloride. — The Pronase- and alkali-treated material was adjusted to 0.03M in NaCl and 0.15% in cetylpyridinium chloride (CPC). After 48 h at room temperature, the precipitate was collected by centrifugation (15,000 *g*, 30 min) and washed three times with 0.15% of CPC in 0.03M NaCl by resuspension and centrifugation. CPC was added to the supernatant solution and washings to a final concentration of 1%. The small amount of precipitate formed was collected and washed as above. The precipitates were combined and fractionally extracted by stirring with 0.2, 0.4, 0.6, 1.0, 1.2, and 2.0M NaCl, utilizing 1×20 ml and 3×10 ml for each extraction; at least one of the extractions at each molarity was done overnight. The CPC in the extracts was removed by dialysis at 40–45° against 2M NaCl followed by distilled water at 4°; the solutions were then filtered and lyophilized.

Enzyme digestions. — The labeled samples (10,000–25,000 d.p.m., ^3H) were mixed with 500 μg of carrier mucopolysaccharide (hyaluronic acid, chondroitin sulfate, or heparan sulfate) and digested with hyaluronidase, chondroitinase, or heparitinase as described below. The carrier mucopolysaccharides were completely degraded under the conditions employed. Testicular hyaluronidase digestion was performed in 700 μl of 0.1M sodium acetate buffer (pH 5.0) and 8mM EDTA in the presence of toluene and chloroform at 37° for 24 h, using 80 units of enzyme. Bacterial hyaluronidase digestion was performed in 0.1M sodium acetate buffer (pH 5.0) at 37° for 26 h, using 150 units of enzyme in a total volume of 150 μl. Leech hyaluronidase digestion was carried out by dissolving the sample in 100 μl of McIlvaine's buffer (pH 5.6) and incubating at 37° for 6 h with the addition of 125 μg of enzyme in 25 μl of buffer at 0, 1, and 2 h. Heparitinase digestions[9] were done in 300 μl of 0.1M sodium acetate buffer (pH 7.0) containing mM calcium acetate at 43° for 24 h. Chondroitinase and chondrosulfatase digestions were performed according to the method of Saito *et al.*[20].

Nitrous acid degradation was done by treating the sample in 100 μl of water with 20 μl of 3M $NaNO_2$ and 20 μl of glacial acetic acid at room temperature for

80 min. Excess of nitrite was destroyed by addition of 50 μl of 3M glycine and, after 60 min at room temperature, the product was lyophilized.

The molecular weights ($\overline{M}_w$) of standard saccharides and unknown samples were determined by sedimentation equilibrium on a Beckman Model E analytical ultracentrifuge by using Rayleigh interference optics. Samples dissolved in an electrolyte, usually 0.5M NaCl, were run at 25° in a three-channel cell at three different concentrations[21]. The fringe patterns were recorded photographically, and the data were read and analyzed directly by a computer-operated system[22].

O.r.d. data were obtained on a Cary Model 60 Spectropolarimeter with samples dissolved in M NaCl; a 10-mm cell was used. Infrared spectra (KBr pellets) were recorded on a Perkin–Elmer 267 spectrophotometer.

RESULTS

Table I presents the results of CPC fractionation of the Pronase digest of the two lots of spent media (MI, 5 l; and MII, 3 l) and of fetal-calf serum (S). Fractions MI 0.2 and MII 0.2, which were eluted from the CPC precipitate with 0.2M NaCl, consisted mainly of a mucin-type sialoglycopeptide which has been characterized[11].

0.4M NaCl Fractions. — MI 0.4 (5.5 mg) was fractionated on a CPG column (1.5 × 115 cm), as illustrated in Fig. 1 (middle). The fractions were combined as indicated, dialyzed, and lyophilized to yield MI 0.4a (1.8 mg) and MI 0.4b (3.2 mg). The distribution of [^{3}H]-activity in the hexosamine component of these fractions is given in Table II. The major portion of MI 0.4b was identified as a mucin-type sialoglycopeptide similar to that present in the 0.2M NaCl eluates. Cellulose acetate electrophoresis of MI 0.4a was done in the two buffer systems; the result in calcium acetate buffer is shown in Fig. 2. Two components were detected with Alcian Blue, but the radioactivity was associated entirely with the spot having the higher mobility.

Separation of the labeled component from the unlabeled contaminant was achieved by chromatography of MI 0.4a on a column (1 × 13 cm) of DEAE Sephadex. After loading of the sample, the column was eluted with a linear gradient of 0.01 → 1.0M pyridine acetate (pH 5.1). Fractions (180 × 1 ml) were collected and aliquots assayed for radioactivity; none was detected in these fractions. The labeled component was recovered from the column by elution with 2M NaCl, dialysis, and lyophilization (MI 0.4a2). The fractions (1–180) were also combined, concentrated, and recovered (MI 0.4a1). The results of cellulose acetate electrophoresis of these two fractions are illustrated in Fig. 2. Fraction (MI 0.4a2) gave only one spot and was free of the unlabeled component (MI 0.4a1). Component MI 0.4a1 was hydrolyzed, and analyzed on a Beckman model 121 amino acid analyzer (60-cm column). In addition to amino acids, both 2-amino-2-deoxyglucose and 2-amino-2-deoxygalactose were present in approximately equal amounts; the component was apparently derived from serum and was not further examined.

The molecular weight of MI 0.4a2 was determined by equilibrium sedimentation to be 92,500; a partial specific volume ($\bar{v}$) for chondroitin sulfate[23] of 0.57 was used

TABLE I

RESULTS OF CPC PRECIPITATION AND SALT ELUTION OF PRONASE-DIGESTED SPENT MEDIA AND FETAL-CALF SERUM

Fraction[a]	*Media I (MI; 5 l)*			*Media II (MII; 3 l)*			*Fetal-calf serum (S; 300 ml)*
	Weight (mg)	^3H *(d.p.m.* $\times 10^{-6}$*)*	^{35}S *(d.p.m.* $\times 10^{-4}$*)*	*Weight (mg)*	^3H *(d.p.m.* $\times 10^{-6}$*)*	^{35}S *(d.p.m.* $\times 10^{-4}$*)*	*Weight (mg)*
0.2M NaCl	7.4	2.28	0.47	1.9	0.64	0.09	0.7
0.4M NaCl	7.8[b]	0.40	1.48	7.1	2.40	1.12	1.1
0.8M NaCl	12.1	1.93	18.83	6.9	0.66	20.00	2.4
1.2M NaCl	2.2	0.69	0.81	0.5	0.33	8.40	0.3
2.0M NaCl	2.0	0.06	0.80	0.9	0.02	2.20	3.8

[a]These fractions are referred to as MI0.4, MII0.4, etc., in the text. [b]Some of this fraction (MI0.4) was lost during work-up of the extract.

for the calculation (Table II). The sample recovered after molecular weight determination was used for o.r.d. measurements. The results are shown in Fig. 3. The sample was then dialyzed against water, KBr (300 mg) was added, and the solution was lyophilized. The resulting powder was dried in a desiccator over P_2O_5, and pressed into a pellet, and the i.r. spectrum was recorded (Fig. 4).

O.r.d. measurements and i.r. spectra were also done on cartilage proteoglycan, hyaluronic acid, and cartilage chondroitin-4-sulfate standards.

Chromatography of the fraction MI 0.4a2 before and after treatment with alkaline borohydride (M $NaBH_4$–0.1M NaOH, 37°, 72 h) gave the results shown in Fig. 5. There was very little change in molecular size on treatment with alkaline borohydride.

Fraction MII 0.4 was also separated into MII 0.4a and MII 0.4b on a CPG column (Fig. 1). MII 0.4a (1.69 mg), consisting of mucopolysaccharide of high molecular weight, was further purified on a column of Sepharose 4B as described

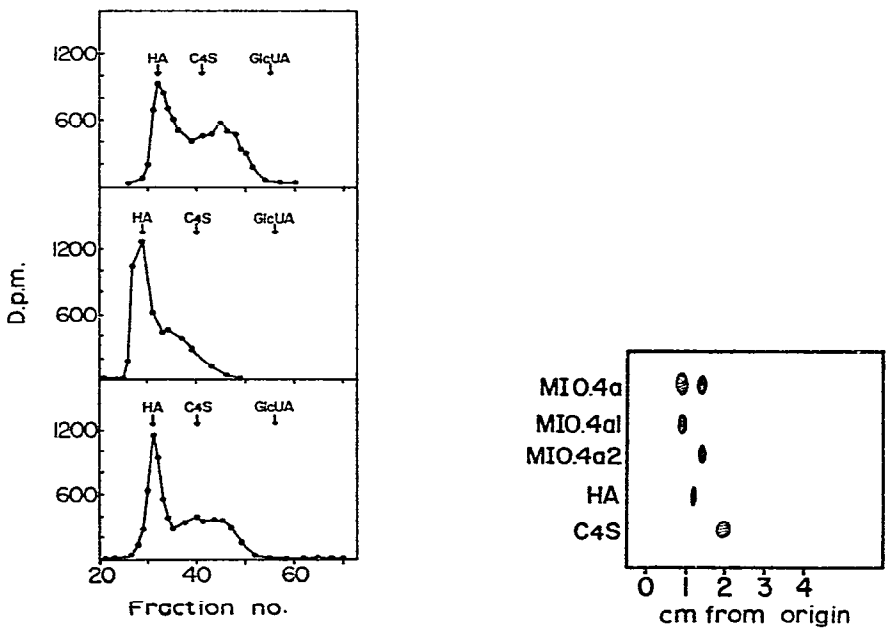


Fig. 1. Fractionation of 0.4M or 0.8M NaCl fractions on a column (1.5 × 115 cm) of CPG 10-240 beads; fractions (3 ml) were collected and analyzed for radioactivity (^3H). The peak-elution position of marker saccharides (HA, vitreous humor hyaluronic acid; C4S, porcine rib-cartilage chondroitin-4-sulfate, and GlcUA, glucuronic acid) are indicated by the arrows. MI0.8 (upper); fractions 29–38 and 39–52 were separately combined and recovered to yield MI0.8a and MI0.8b, respectively. MI0.4 (middle); fractions 26–32 and 33–45 were recovered to yield MI0.4a and MI0.4b, respectively. MII0.8 (lower); fraction 27–33 and 34–52 were combined and recovered to yield MII0.8a and MII0.8b, respectively.

Fig. 2. Cellulose acetate electrophoresis in calcium acetate buffer of MI0.4a before and after (MI0.4a1 and MI0.4a2) fractionation on a column of DEAE Sephadex; details are given in the text. Shaded spots stained more intensely with Alcian Blue than the dotted spots.

TABLE II

ANALYTICAL DATA FOR VARIOUS MEDIA SUBFRACTIONS

Fraction	*Weight (mg)*	*GlcN*[a]	*GalN*[a]	*Mol. Wt.*[b]
MI0.4a	1.80	2.8	97.2	47,400
MI0.4b	3.21	8.8	91.2	~15,000
MI0.4a1[c]	0.42	n.d.[d]	n.d.	n.d.
MI0.4a2[c]	1.17	0	100.0	92,500[e]
MII0.4a[f]	1.69	n.d.	n.d.	120,600
MI0.8a	1.38	5.8	94.2	80,000
MI0.8b	1.53	71.5	29.5	~15,000
MII0.8a[g]	0.80	3.5	96.5	121,000
MII0.8b	1.40	67.5	32.5	~15,000

[a]The hexosamine values are as percentages of the tritium label found for 2-amino-2-deoxy-glucose and -galactose. [b]Based on the behavior of the labeled components relative to standard mucopolysaccharides during chromatography on CPG (MI0.4b, MI0.8b, and MII0.8b) or by sedimentation equilibrium analysis (others). [c]Obtained by fractionation of MI0.4a on DEAE Sephadex; see Results for details. [d]n.d., Not determined. [e]Average of two separate determinations (88,000 and 97,000). [f]Determined after purification on Sepharose 4B. [g]Determined after treatment with DNase, RNase, and Pronase, and purification by chromatography on Sephadex G200 and Sepharose 4B.

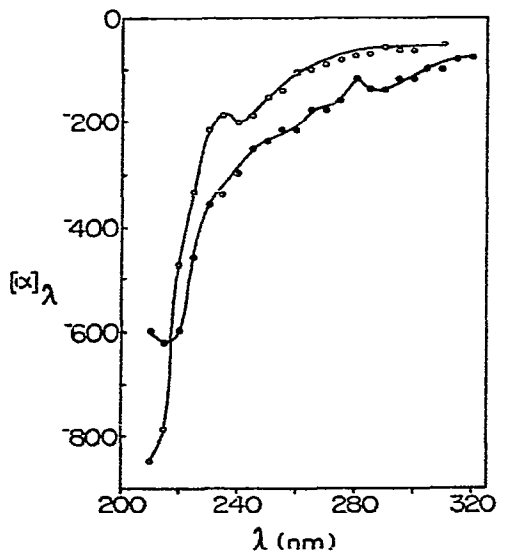


Fig. 3. O.r.d. curves for fractions of mouse melanoma chondroitin sulfate: —○—, MI0.4a2; and —●—, MI0.8a.

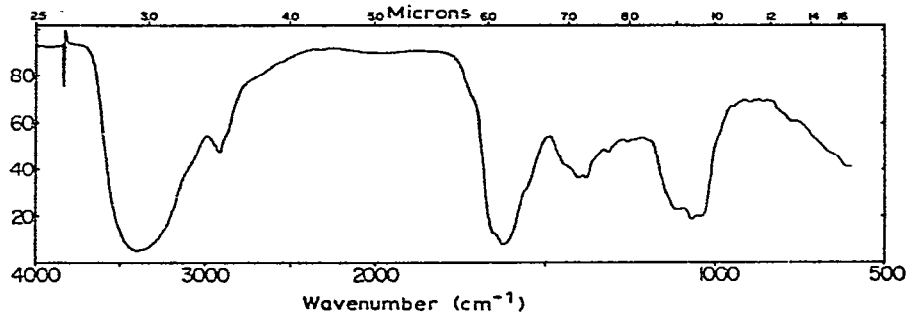


Fig. 4. Infrared spectrum for fraction MI0.4a2 of mouse melanoma chondroitin sulfate.

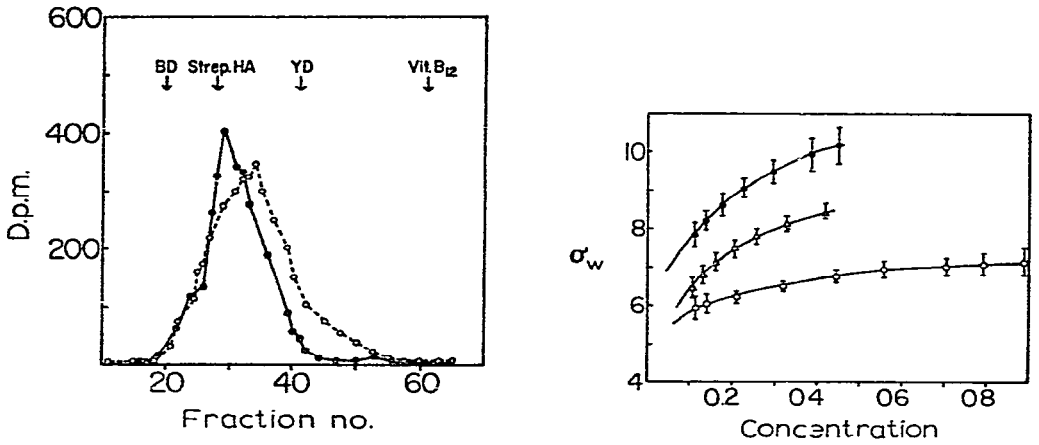


Fig. 5. Gel filtration of mouse melanoma fraction MI0.4a2 before (—●—) and after (—○—) treatment with M $NaBH_4$–0.1M NaOH (37° for 72 h) on a column of Sepharose 4B (2 × 60 cm). Elution positions of calibration standards are indicated by arrows: BD, blue dextran; YD, yellow dextran; Vit. B_{12}, vitamin B_{12}; and Strep. HA, streptococcal hyaluronic acid. The fractions were analyzed for ^3H activity.

Fig. 6. Molecular moments for fraction MII0.4a of mouse melanoma mucopolysaccharide in 0.5M NaCl: r.p.m. = 15,000; loading concentration channel A (●), 0.5 mg/ml; channel B (△), 0.25 mg/ml; and channel C (○), 0.125 mg/ml.

below for MII 0.8a, and the main peak was used for determination of molecular weight. A molecular weight ($\overline{M}_w$) of 120,600 ±5800 was estimated (Fig. 6); a two-species plot[24] showed the presence of aggregates up to trimers.

0.8M NaCl Fractions. — Fractionation on a CPG column gave the elution pattern shown in Fig. 1 (upper); MI 0.8a (1.38 mg) and MI 0.8b (1.53 mg) were obtained. Hexosamine analyses of these fractions are given in Table II. Cellulose acetate electrophoresis of MI 0.8a was done in two buffer systems. In pyridine formate buffer, the major spot moved slightly ahead of hyaluronic acid, and the second spot,

present only in trace amounts, had a mobility similar to that of heparan sulfate (Fig. 7). O.r.d. measurements were done on MI 0.8a (Fig. 3), which had $[\alpha]_{280}$ −120°; porcine rib-cartilage chondroitin sulfate has $[\alpha]_{280}$ −219°, and vitreous humor hyaluronic acid −520°. This fraction was digested with Pronase for 48 h at 40°, with two additions of the enzyme at 0 and 24 h. The gel-filtration pattern on CPG of the digested material was the same as that of the undigested fraction. This indicates that the molecule does not consist of several saccharide chains attached to a Pronase-susceptible peptide, in agreement with earlier observations on material isolated from the nuclei of melanoma cells[3].

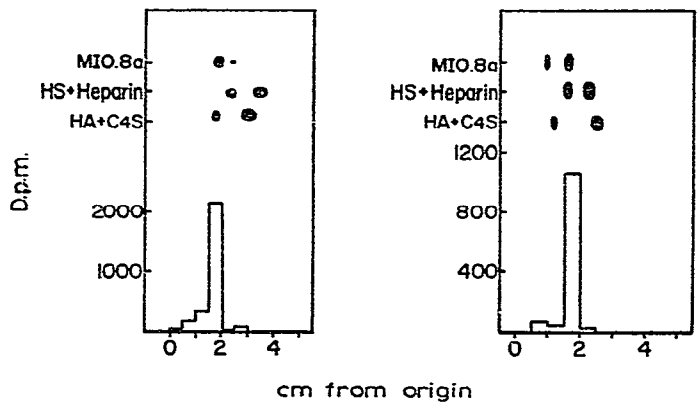


Fig. 7. Cellulose acetate electrophoresis of mucopolysaccharide fraction MI0.8a from mouse melanoma in pyridine acetate (left) and calcium acetate (right) buffers. The unknown sample was run in two lanes; after electrophoresis, one lane was assayed for radioactivity (see Methods) and the remainder of the strip was stained with Alcian Blue.

This fraction was digested with testicular hyaluronidase and chondroitinase AC, and the digests were examined by chromatography on CPG columns. The material was completely susceptible to both of these enzymes. The fraction was also susceptible to bacterial hyaluronidase, but the fragments obtained were larger than tetrasaccharides and disaccharides, as illustrated for the nuclear material[3]. These results are in agreement with a polysaccharide having 50% or less of the disaccharide repeating-units sulfated, more or less at random. Chondroitinase AC and chrondroitinase AC plus chondro-4-sulfatase digestion of this fraction, followed by paper chromatography as illustrated in Fig. 8, indicates that the sulfate ester group is mainly at position 4 of the 2-amino-2-deoxygalactose moiety.

In order to identify the uronic acid and neutral sugars by g.l.c., and the hexosamine by the ninhydrin-degradation method, this fraction was hydrolyzed under two different conditions and the products were separated as shown in Fig. 9. The uronic acid was identified as glucuronic acid; there was no evidence for the presence of iduronic acid, thereby eliminating the possibility of a chondroitin sulfate–dermatan sulfate co-polymer[25]. The results of the neutral sugar analysis were not very clear, as

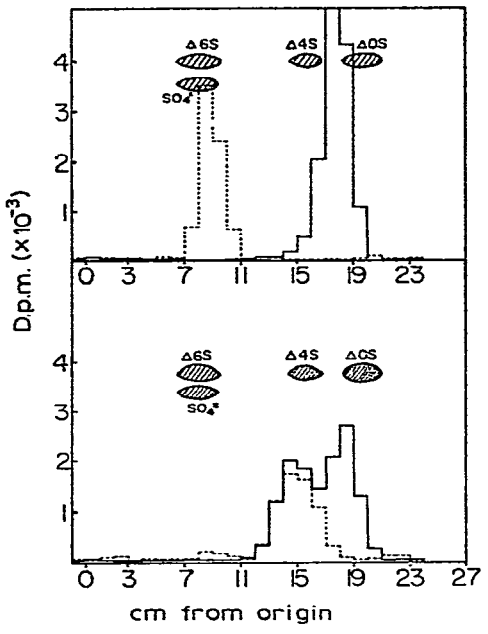
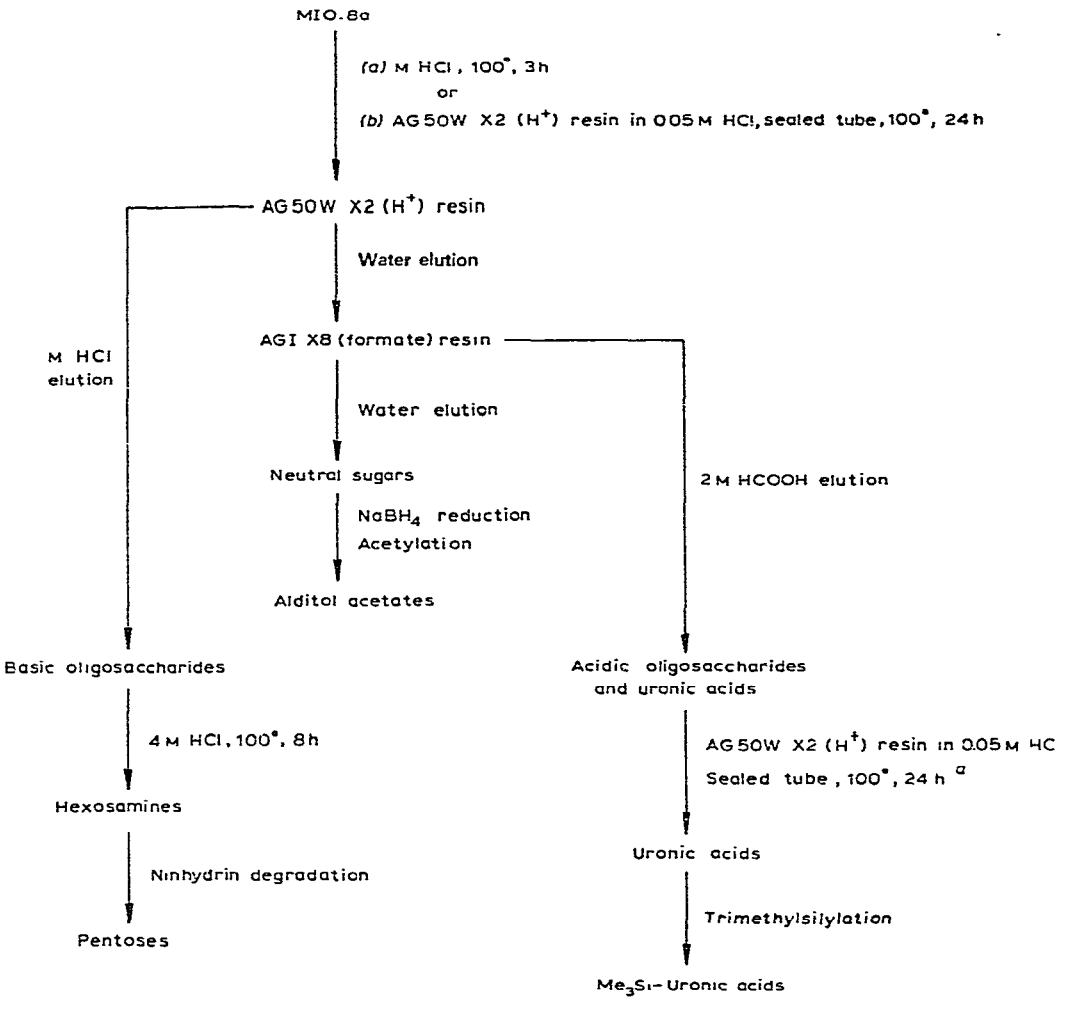


Fig. 8. Paper chromatography (1-butanol–acetic acid–M ammonium hydroxide, 2:2:1; 18 h) of chondroitinase AC (lower) and chondroitinase AC and chondro-4-sulfatase (upper) digests of fraction MI0.8a. The positions of reference compounds are indicated: Δ4S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose; Δ6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔOS, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose.

there were several unidentifiable peaks. However, small peaks of xylose and galactose were detectable, but their stoichiometry could not be assessed; the presence of these sugars needs to be confirmed by independent methods. Polysaccharide chains of molecular weight ~120,000, if synthesized *via* glycosyl-serine initiation, would be expected to have only one xylose residue per 600 monosaccharide residues. Thus, larger amounts of samples are needed for quantitation of the neutral sugars. The hexosamine fraction was degraded with ninhydrin[26], and the products were examined by paper chromatography (ethyl acetate–pyridine–water, 9:3:2) with arabinose and lyxose as reference sugars. The labeled material had a mobility identical to that of lyxose, confirming the identity of the hexosamine as 2-amino-2-deoxygalactose; arabinose was not detected.

A major portion of MI 0.8b was susceptible to nitrous acid treatment. As 72% of the hexosamine in this fraction was 2-amino-2-deoxyglucose, it can be concluded that heparan sulfate is the major constituent of MI 0.8b. The remainder is apparently chondroitin sulfate[3] of molecular weight 10–15,000. When MI 0.8b was treated with heparitinase and chromatographed on CPG, ~60% of the ^{3}H-radioactivity was eluted in the region of low molecular weight, a result in agreement with the nitrous acid degradation.



[a] Sample hydrolyzed under condition *(b)* was directly trimethylsilylated.

Fig. 9. Scheme for degradation and isolation of neutral sugars, uronic acids, and hexosamines from mouse melanoma fraction MI0.8a.

Fraction MII 0.8 was separated into fractions of higher and lower molecular weight on a CPG column (1.5 × 115 cm) (Fig. 1, lower). The fraction of higher molecular weight (MII 0.8a, 2.2 mg) was further purified as follows. It was dissolved in 50mM Tris–HCl buffer (pH 7.4) containing 10mM Mg^{2+}, and treated with deoxyribonuclease I and ribonuclease at 37° for 48 h. The product, recovered by dialysis and freeze-drying, was then dissolved in 50mM Tris–HCl buffer (pH 8.0) containing

10mM Ca^{2+}, and digested with Pronase at 40° for 3 days in the presence of toluene; enzyme was added at time 0 and at 24 h. The digest was chromatographed on a column of Sephadex G-220, and the peak containing all the radioactivity was recovered and chromatographed on a column of Sepharose 4B (Fig. 10). The component in the sharp portion of the peak (fractions 26–39) was recovered (0.72 mg). Cellulose acetate electrophoresis of this peak in calcium acetate buffer gave a single Alcian Blue-staining spot of mobility intermediate between that of hyaluronic acid and chondroitin-4-sulfate. The molecular weight of this purified material was estimated to be 121,000 by sedimentation equilibrium analysis; a two-species plot gave evidence for the presence of aggregates (trimers). Digestion of a portion of this fraction with leech hyaluronidase, with chromatography on a CPG column, gave the results shown in Fig. 11a. In parallel experiments, it was found, as expected, that the enzyme digested hyaluronic acid, but had no action on chrondroitin-4-sulfate from porcine rib-cartilage. The action of leech hyaluronidase on proteoglycan is illustrated in Fig. 11b. There is a slight change in the molecular size distribution, suggesting there may be a few leech hyaluronidase-susceptible bonds in proteoglycan.

Serum fraction S 0.8. — This fraction was also digested with deoxyribonuclease, ribonuclease, and finally Pronase, as described above. The digested sample was chromatographed on a column of Sepharose 4B, and the fractions (26–39) corresponding to the peak in Fig. 10 were combined, dialyzed, and lyophilized to yield 0.59 mg of material. Similarly, fractions 16–25 and 40–70 were also recovered, yielding 0.12 and 1.80 mg, respectively. Cellulose acetate electrophoresis of these fractions in pyridine acetate and calcium acetate buffers did not show any Alcian Blue-staining spots when spotted in the usual concentrations. When fifteen times the usual amount was subjected to electrophoresis, very diffuse areas were stained with Alcian Blue. Fractions 26–39 showed staining in an area of lower mobility than the cor-

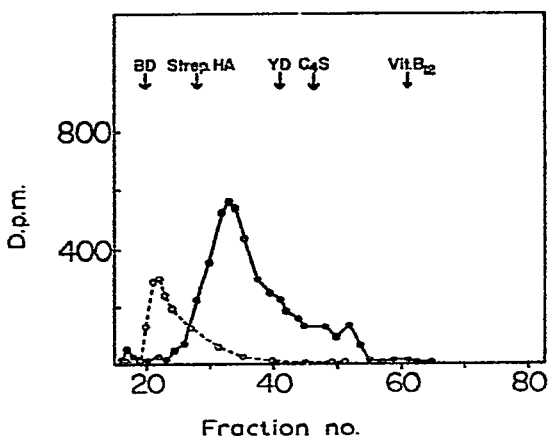


Fig. 10. Chromatography of MII0.8a (—●—) on a column (2 × 60 cm) of Sepharose 4B after treatment with deoxyribonuclease, ribonuclease, and Pronase. Elution pattern of vitreous humor hyaluronic acid (—○—) and the peak-elution positions of other standards (see Figs. 1 and 5) are shown.

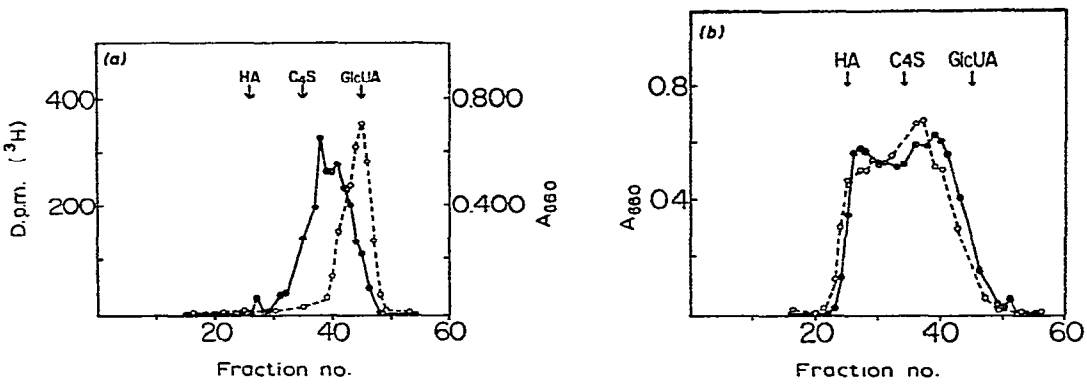


Fig. 11. (*a*) Chromatography of fraction MII0.8a (—●—) and hyaluronic acid (—○—) on a column (0.9 × 61 cm) of controlled-pore glass (CPG) 10-240 beads after digestion with leech hyaluronidase. Abbreviations are as in Fig. 1. Before treatment, both samples were eluted at the position indicated by HA. (*b*) Elution patterns of cartilage proteoglycan, before (—●—) and after (—○—) digestion with leech hyaluronidase, on the same CPG column.

responding fractions from MII 0.8a. Fractions 40–70 from serum, however, gave a streaky spot with the mobility of rib-cartilage chondroitin-4-sulfate.

DISCUSSION

The purity of the labeled polyanionic components isolated from culture media was established by the criteria that the radioactivity and Alcian Blue-staining material co-migrated on cellulose acetate electrophoresis in two different buffer systems. Chemical characterization of the components is based mainly on the distribution of radioactivity in hexosamines, and on the susceptibility of the labeled component to enzymes. In addition, the charge properties, as determined by the elution from the cetylpyridinium chloride precipitate, and by the behavior on electrophoresis, confirm the identity of the components. The identification of the major mucopolysaccharide components as a family of chondroitin-4-sulfates of high molecular weight, and the minor components as heparan sulfate and chondroitin sulfates, is in agreement with previous results[3,8].

The susceptibility of the chondroitin sulfate of high molecular weight to leech hyaluronidase (Fig. 11) was surprising. This enzyme is an endo-glucuronidase, and is considered specific for linkages between 2-acetamido-2-deoxy-D-glucose and D-glucuronic acid[27]. It is possible that the melanoma chondroitin sulfate may have a few 2-acetamido-2-deoxy-D-glucose residues in the chain. Cleavage of only a few bonds in the molecule could cause the change in molecular size observed on CPG columns, whereas the presence of 2% of 2-amino-2-deoxy-D-glucose (3–4 residues) might not be detected by the analytical techniques described. There was insufficient material to further investigate this possibility.

Physical measurements were carried out on the chondroitin of high molecular weight, and the chondroitin-4-sulfate having ~50% sulfation, which eluted from the

CPC precipitate with 0.4 and 0.8M NaCl, respectively. Fig. 6 shows the results of a representative sedimentation-equilibrium analysis of these samples. The sample shows considerable polydispersity in molecular weights, as each concentration gave a different curve. The weight-average molecular weights ($\bar{M}_w$) calculated from several experiments were in the range of 80,000–120,000. The elution pattern of the labeled component from a column of Sepharose 4B (Figs. 5 and 10), in relation to streptococcal and vitreous humor hyaluronic acids, is in agreement with the above values[28]. The lower values of 80,000–95,000 may be due to the presence of contaminants of low molecular weight in these samples. For example, the sample MI 0.4a, which still had a serum component (MI 0.4a1) of low molecular weight as a contaminant, had $\bar{M}_w$ 47,000. The apparent polydispersity of the molecules could be partly due to associative interactions. A two-species plot indicated the presence of aggregates (monomer–trimer systems) of these polyanionic molecules, in agreement with earlier observations on cartilage proteoglycan[24]. It can be concluded that the molecular size of the melanoma chondroitin sulfate is in the range of 90,000–120,000, compared to 13,000 for the rib-cartilage product[24]. As the melanoma product was not susceptible to further Pronase digestion or to degradation with alkaline borohydride, it is more likely to be a single polysaccharide chain and not several smaller chains attached to a common peptide.

O.r.d. of the melanoma component (Fig. 3) was characteristic of mucopolysaccharides with a trough at 210–215 nm. The o.r.d. spectrum of chondroitin-4-sulfate chains from cartilage showed a trough at 220 nm, and that of vitreous humor hyaluronic acid at 215 nm. The infrared spectrum of MI 0.4a2 resembled that of cartilage chondroitin-4-sulfate, except for the absence of absorption in the sulfate region between 1230–1250 cm^{-1}. The lack of sulfate absorption is expected, as the fraction eluting with 0.4M NaCl from the CPC precipitate is low in sulfate. The $^{35}S/^{3}H$ ratio in MI 0.4 is 0.037, compared to 0.098 for MI 0.8 (Table I). The infrared spectra of hyaluronic acid and of cartilage proteoglycan were distinguishable from that of the melanoma component.

It has been reported that fetal-calf serum contains acid mucopolysaccharides in addition to large quantities of glycoproteins[29]. The chondroitin sulfate of high molecular weight identified above was metabolically labeled with 2-amino-2-deoxy-D-glucose-*t* and $^{35}SO_4^{2-}$ as illustrated, for example, by the production of disaccharides labeled with ^{3}H and ^{35}S on digestion of MI 0.8a with chondroitinase AC (Fig. 8). This, together with the fact that it was also isolated from the cell pellet[8] and nuclei[3], is inconsistent with its being a serum component. The possibility of a serum contaminant co-purifying with the melanoma chondroitin sulfate and leading to misinterpretation of the physical data was tested by carrying out a control experiment with fetal-calf serum. The fractions eluting with 0.4 and 0.8M NaCl were isolated from fetal-calf serum by procedures identical to those used for spent media. However, on fractionation of the 0.8M fraction on Sepharose 4B, the major portion was eluted in the region of low molecular size and behaved in cellulose acetate electrophoresis like cartilage chondroitin sulfate. The material (~0.6 mg) eluted in the same region

as the melanoma chondroitin sulfate may not be a polyanionic component because, even at fifteen times the usual concentration, it failed to stain with Alcian Blue when examined by cellulose acetate electrophoresis.

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