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STRUCTURE OF DERMATAN SULFATE. VI. THE USE OF CETYLPYRIDINIUM CHLORIDE-CELLULOSE MICROCOLUMNS FOR DETERMINATION OF THE HYBRID STRUCTURE OF DERMATAN SULFATES*

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ABSTRACT

A variety of dermatan sulfate samples have been analyzed by the cetylpyridinium chloride–cellulose microcolumn technique before and after treatment elution with testicular hyaluronidase. It was found that "neutral magnesium chloride elution profiles" reflected the molecular-size polydispersity of the material obtained after hyaluronidase degradation. However, "acid magnesium chloride elution profiles" were not influenced by molecular size to any large extent; instead, the proportions of L-iduronic and D-glucuronic acid as well as the sulfate content seemed to be the principal parameters involved in these elution profiles. Barium acetate–ethanol cellulose elution profiles, finally, were found to separate various dermatan sulfate samples almost entirely according to uronic acid composition. Thus, by comparing elution profiles of unknown samples to those of well-characterized dermatan sulfate polymers, sufficient data were obtained to permit a formulation of the general hybrid properties of beef-aorta and sclera dermatan sulfate as well as of the dermatan sulfate obtained from human knee-joint capsule.

INTRODUCTION

Previous studies have demonstrated that dermatan sulfates of skin[1,2], umbilical cord[3], and intestinal mucosa[4] are hybrid polymers composed of three principal types of repeating units: (1→3)-*O*-(2-acetamido-2-deoxy-*β*-D-galactopyranosyl 4-sulfate)-(1→4)-*β*-D-glucopyranuronosyl (A type), (1→3)-*O*-(2-acetamido-2-deoxy-*β*-D-galactopyranosyl 4-sulfate)-(1→4)-*α*-L-idopyranuronosyl (B type), and (1→3)-*O*-(2-acetamido-2-deoxy-*β*-D-galactopyranosyl 6-sulfate)-(1→4)-*β*-D-glucopyranuronosyl (C type). Pig-skin dermatan sulfate is composed almost exclusively of 4-sulfated units[2] (AB hybrid), whereas umbilical-cord dermatan sulfate contains both 4- and

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6-sulfated hexosamine moieties[3] (BC hybrid). In these polymers the glucuronic acid-containing disaccharide units are generally assembled in clusters of two or three to form, on an average, one or two hyaluronidase-susceptible linkages per chain. In both skin and umbilical-cord dermatan sulfate, the glucuronic acid-containing sections appear to be distributed at random along the polysaccharide chain[2,3]. However, the glucuronic acid-containing repeating units of intestinal-mucosa dermatan sulfate are located exclusively at the nonreducing terminal portion of the polymer[4].

Analyses of the hybrid nature of dermatan sulfates by conventional methods is quite laborious and requires a considerable amount of material. Thus, studies of dermatan sulfates of limited availability entail the use of a micromethod. The cetylpyridinium chloride (CPC)–cellulose microcolumn technique[5] was therefore applied to the analysis of various dermatan sulfate samples before and after treatment with testicular hyaluronidase. The results presented in this paper indicate that a combination of the so-called "neutral magnesium chloride elution profiles" and "acid magnesium chloride elution profiles" together with barium acetate–ethanol cellulose elution profiles will provide information concerning the length of the polysaccharide fragments, the sulfate distribution, and the relative proportions of L-iduronic acid and D-glucuronic acid residues in these fragments.

The CPC–cellulose microprocedure has previously been used in this laboratory for studying the depolymerisation of various glycosaminoglycans by testicular hyaluronidase[6].

EXPERIMENTAL

Materials. — Beef aorta and eyes as well as horse aorta were obtained from the local slaughterhouse. Specimens of human knee-joint capsule were obtained through the courtesy of Dr. Bo Lundberg, Department of Orthopedic Surgery, Malmö General Hospital, Malmö, Sweden. Chondroitin 4-sulfate was prepared from beef nasal septum according to Antonopoulos *et al.*[5,7]. Testicular hyaluronidase (20,000 i.u. per mg) was purchased from AB Leo, Hälsingborg, Sweden. Crystalline papain (25 to 30 units per mg of protein) was obtained from a crude preparation (Type II; Sigma) according to the procedure of Kimmel and Smith[8]. Cetylpyridinium chloride (CPC) was a gift from AB Recip, Stockholm, Sweden. Blue dextran 1000 was a product of Pharmacia Fine Chemicals, Uppsala, Sweden. Sodium [^{35}S]sulfate was obtained from the Radiochemical Centre, Amersham, England.

Preparation of dermatan sulfates. — Dermatan sulfate was prepared from acetone-dried, ground tissue powder by digestion with papain and fractionation with CPC and calcium acetate–ethanol as described previously[1,2,4,9]. The 0–25% ethanol fractions were used in the present study, if not stated otherwise. Analytical data are shown in Table I.

Fractionation of hyaluronidase-treated dermatan sulfate. — Beef-aorta dermatan sulfate was treated with testicular hyaluronidase and the polysaccharide material was isolated by ethanol precipitation as described previously[1]. The enzyme-treated dermatan sulfate (80 mg) was then fractionated on a column (1 × 100 cm) of cellulose equilibrated with 1% (w/v) CPC-0.005M sodium sulfate[7]. The column was eluted with 2–3 bed volumes of 0.6M magnesium chloride–0.1M acetic acid–0.05% CPC (I), 0.9M magnesium chloride–0.1M acetic acid–0.05% CPC (II), and 2.0M sodium chloride–0.05% CPC (III). The effluents were collected in 10-ml fractions, and the polysaccharide was detected by the orcinol method[1]. The polysaccharide was isolated by precipitation with 3 vol. of ethanol. The sediments were washed with abs. ethanol and ether, and dried *in vacuo* over phosphorous pentoxide. The yields were: I, 54 mg; II, 10 mg; and III, 7 mg. Analytical data are shown in Table II.

Methods. — Hexosamine was determined by the Elson–Morgan procedure[10] after hydrolysis with 6M hydrochloric acid for 8 h in a boiling water bath and subsequent removal of the acid in a desiccator over sodium hydroxide pellets under reduced pressure[5]. Hexuronic acids were estimated by the carbazole method of Dische[11] and by the orcinol method[12] using a 20-min boiling time. Sulfate was determined by the modified benzidine method of Antonopoulos[13]. I.r. spectra were recorded with a Unicam SP 200 spectrophotometer on potassium bromide pellets.

Gel chromatography of dermatan sulfate samples was carried out on columns (1.2 × 190 cm) of Sephadex G-200 (fine), eluted with 0.2M sodium chloride–10% ethanol at a rate of 10 ml per h. Aliquots of the fractions (2 ml) were analyzed by the orcinol method[12]. The columns were calibrated with blue dextran and sodium [^{35}S]sulfate in order to determine the void volume (v_0) and the total volume (v_t), respectively[14]. The elution volume (v_e) of each preparation was defined as the position of the maximum of the eluted peak.

Dermatan sulfate samples were analyzed by the CPC–cellulose microcolumn technique of Antonopoulos *et al.*[5]. The columns were eluted with either neutral or acid (0.1M acetic acid) magnesium chloride solutions[15]. The barium acetate–ethanol cellulose column technique of Gardell[16] was adapted to the microscale as described for the CPC–cellulose microcolumn technique[5].

RESULTS AND DISCUSSION

CPC-solubility profiles at neutral pH. — A number of dermatan sulfate samples from various sources (Table I) were analyzed by the CPC–cellulose microprocedure. The solubility curves obtained at neutral pH are shown in Fig. 1. It was found that when the logarithm of the critical electrolyte concentration, CEC, (i.e. the salt concentration which solubilizes 50% of the total polysaccharide) was plotted against $1/M_v \times 10^5$ (samples 7–12) according to Laurent and Scott[19] a straight line was obtained (Fig. 2).

Another set of dermatan sulfate samples (Nos. 1, 2, 5, and 6 of Table I) was subjected to gel chromatography (Fig. 3a–d) and their K_{av} values[14] were compared with their solubility characteristics in the CPC–cellulose column technique. The plot (Fig. 4) indicates good correlation between the log CEC and $1/K_{av}$. It may thus be

TABLE I

ANALYSES OF VARIOUS DERMATAN SULFATE SAMPLES

Sample No.	Source	Treatment	Hexosamine, %	Uronic acid (carbazole), %	Carbazole / orcinol	Sulfur / hexosamine[a]	K_{av}	Molecular weight, M_v[b]	Critical electrolyte concentration[c]
1	Sclera	—	21.9	12.9	0.43	1.00	0.19		0.61
2	Sclera	H[d]	27.6	16.6	0.32	0.97	0.53		0.44
3	Beef aorta	—	25.0	15.4	0.50	0.50			0.47
4	Beef aorta	H[d]	22.3	12.6	0.47	0.66			0.36
5	Joint capsule	—	27.8	14.0	0.35	0.51	0.31		0.46
6	Joint capsule	H[d]	27.3	13.1	0.33	0.63	0.78		0.40
7	Mucosa[e]	—	24.6	13.3	0.26	0.99		50,000	0.51
8	Mucosa	H[d]	20.5	11.5	0.29	1.16		40,000	0.50
9	Pig skin[f]	—	25.2	14.2	0.34	1.06		25,000	0.46
10	Pig skin	H[d]	21.8	15.2	0.32	1.24		15,000	0.40
11	Pig skin	H-18[g]	22.9	14.2	0.29	1.28		16,000	0.41
12	Pig skin	H-25[h]	21.1	10.4	0.24	1.04		13,000	0.36
13	Pig skin	H-2.0[i]	24.7	14.7	0.29	1.16		16,000	
14	Pig skin	H-4.0[i]	20.5	12.0	0.29	1.52		20,000	
15	Horse aorta	25-25[j]	23.5	19.3	0.47	0.96		40,000	
16	Horse aorta	25-50[j]	22.2	20.4	0.58	1.02		40,000	
17	Horse aorta	35[j]	24.0	21.5	1.02	1.06		50,000	

[a] Molar ratio; a 15%-destruction of hexosamine during hydrolysis is assumed. [b] Viscosity-average molecular weight (M_v) was calculated according to Mathews equation[17]: $\eta_{sp}/C = [\eta] = 3.1 \times 10^{-4} \times M_v^{0.74}$. Determination of the reduced viscosity (η_{sp}/C) was performed as described by Mathews and Dorfman[18] at a polysaccharide concentration of 2 mg per ml of 0.15M sodium chloride–0.1M acetate buffer, pH 5.0. At this concentration, the reduced viscosity is close to the intrinsic viscosity[17,18]. [c] Critical electrolyte concentration (CEC) is defined as the salt concentration (M) which solubilizes 50% of the total polysaccharide material in the CPC–cellulose microcolumn technique. [d] H, Hyaluronidase treated sample; the dermatan sulfate samples were treated with testicular hyaluronidase and the polysaccharide material was re-isolated as previously described[1]. [e] For preparative procedure, see ref. 4. [f] For preparative procedure, see ref. 9. [g] Fraction II, 2-18-H-18 (ref. 2). [h] Fraction II, 2-25-H-25 (ref. 2). [i] Sample 10 (3.5 g) was fractionated on a column (4.5 × 50 cm) of AG 1-X2 (Cl^-, 200–400 mesh). The column was eluted with 1.0, 1.5, 2.0, and 4.0M sodium chloride. Samples 13 and 14 correspond to the material eluted in the last two fractions. Their yields were 1.6 and 0.5 g, respectively. Further details concerning these two samples will be published elsewhere. [j] Horse-aorta galactosaminoglycans (*i.e.* material precipitated with CPC in the presence of 0.5M sodium chloride, see also ref. 1, 2, 4, 9) were fractionated with calcium acetate–ethanol. The material precipitated with 25% ethanol was reprecipitated with 25% ethanol (80% yield, sample 15); the soluble material from this reprecipitation was precipitated with 50% ethanol (sample 16). The soluble material from the first precipitation was precipitated with 35% ethanol (sample 17).

stated that the neutral magnesium chloride profile of dermatan sulfate reflects the molecular size polydispersity of the polymer. These relationships have previously been demonstrated for chondroitin 4-sulfate[19,20], keratan sulfate[19], and heparin[21].

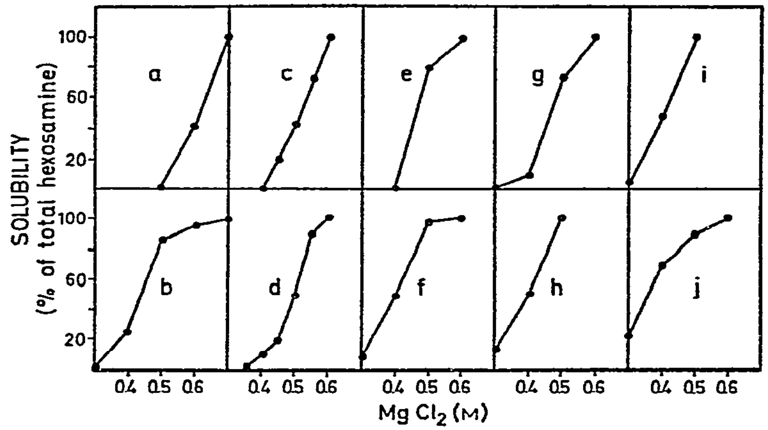

Fig. 1. Solubility curves of the cetylpyridinium (CP)–complexes of various dermatan sulfates: Dermatan sulfate from beef sclera, (a) before and (b) after treatment with testicular hyaluronidase; dermatan sulfate from hog-intestinal mucosa, (c) before and (d) after hyaluronidase treatment; dermatan sulfate from pig skin[9], (e) before and (f) after enzyme treatment; dermatan sulfate from knee-joint capsule, (g) before and (h) after enzyme treatment; and, finally, two dermatan sulfate preparations described elsewhere[2], (i) 11:2-18-H-18 and (j) 11:2-25-H-25. The solubility curves were constructed by integration of the data obtained from solubility profiles[5].

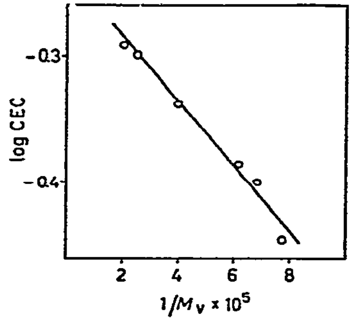

Fig. 2. Relationship between the critical electrolyte concentration (log CEC) and the molecular size ($1/M_v \times 10^5$) of various dermatan sulfate preparations. CEC was obtained from the data shown in Fig. 1 and was defined as the magnesium chloride concentration (M) which solubilized 50% of the total polysaccharide. M_v is the viscosity average molecular-weight[1].

CPC-solubility profiles at acid pH. — It has been shown previously[7,15] that the solubility of dermatan sulfate–cetylpyridinium complexes varies with pH. Indeed, by the use of acid (0.1M acetic) magnesium chloride solutions, it was possible to separate dermatan sulfate from chondroitin sulfates[15]. This separation would appear to be due to the fact that, at pH 2–3, L-iduronic acid and D-glucuronic acid residues are ionized to a different degree[22–24]. Whereas solubility studies performed at neutral pH indicate the molecular-size polydispersity of the material, "acid magnesium chloride

TABLE II

ANALYSES OF DERMATAN SULFATE FRACTIONS FROM BEEF AORTA OBTAINED BY HYALURONIDASE DIGESTION AND CPC–CELLULOSE COLUMN CHROMATOGRAPHY

Fraction	*Hexosamine, %*	*Uronic acid (carbazole), %*	*Carbazole / orcinol*	*Sulfur / hexosamine*[a]
I	17.3	15.9	0.53	0.67
II	21.3	14.2	0.43	0.72
III	22.2	14.4	0.24	0.60

[a] Molar ratio; a 15%-destruction of hexosamine during hydrolysis is assumed.

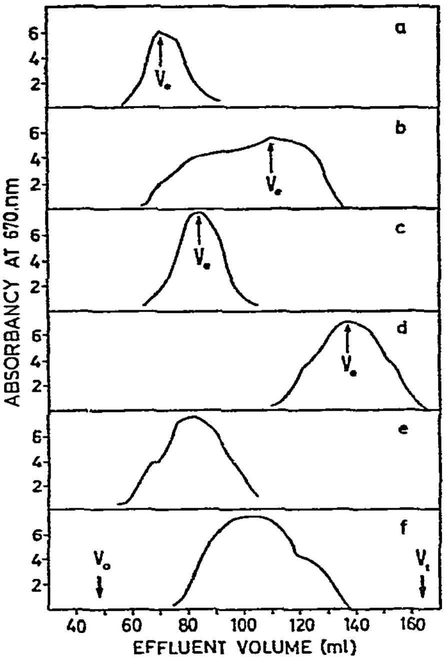

Fig. 3. Gel chromatography of various dermatan sulfate samples: Dermatan sulfate from beef sclera, (a) before and (b) after hyaluronidase treatment; dermatan sulfate from knee-joint capsule, (c) before and (d) after enzyme treatment, dermatan sulfate from beef aorta: (e) Fraction II and (f) Fraction III (Table II). V_0, void volume; V_e, elution volume; V_t, total volume.

elution profiles" may thus be expected to reflect, in addition, the proportions of L-iduronic and D-glucuronic acid residues in the polymer. Furthermore, the degree of sulfation might also be expected to play a role in determining the solubility characteristics at acid pH.

In order to investigate these points, three sets of dermatan sulfate samples were examined. In each set one of the three parameters was varied and the others were kept as constant as possible.

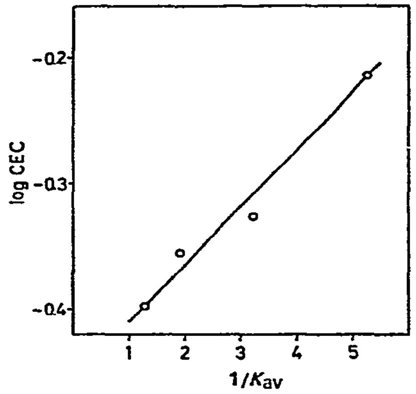

Fig. 4. Relationship between the critical electrolyte concentration (log CEC) and the molecular size ($1/K_{av}$) of various dermatan sulfate preparations. K_{av} was obtained from gel-chromatography experiments[14].

Since the proportions of L-iduronic and D-glucuronic acid can be assessed from the carbazole-to-orcinol (C/O) ratios[25], dermatan sulfate samples possessing C/O ratios ranging from 0.26 to 1.02 (samples 7, 15, 16, and 17 in Table I) were subjected to CPC–cellulose microcolumn fractionation using "acid magnesium chloride

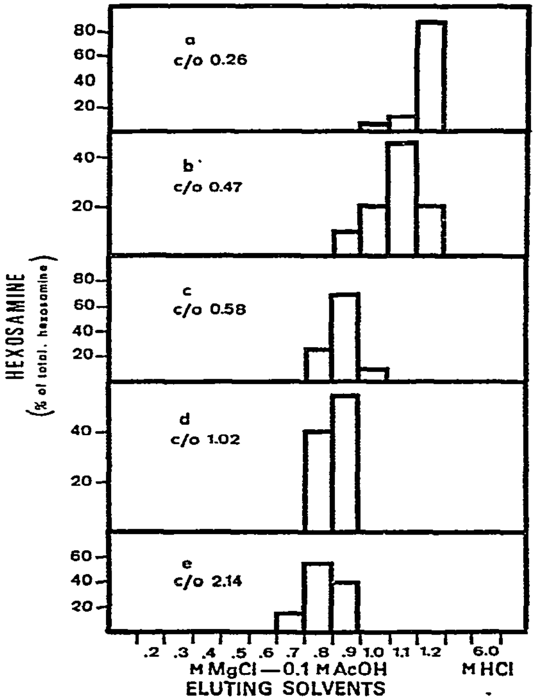

Fig. 5. CPC–cellulose microcolumn elution profiles ("acid magnesium chloride elution profiles") of (a) mucosa dermatan sulfate (sample 7 in Table I); (b), (c), and (d) horse-aorta dermatan sulfates (samples 15, 16, and 17, respectively); and (e) chondroitin 4-sulfate. C/O, carbazole to orcinol ratio.

eluents". As shown in Fig. 5, elution profiles obtained at low pH reflected the L-iduronic to D-glucuronic acid ratio of the material; i.e. the higher the L-iduronic acid content, the higher was the apparent CEC. Dermatan sulfates having C/O ratios between 0.26 and 0.58 could be distinguished by this technique, whereas samples having C/O ratios above 0.6 were essentially indistinguishable from each other. Thus, hybrid dermatan sulfates having more than 20% of the total uronic consisting of D-glucuronic acid cannot be separated from chondroitin sulfate by this procedure.

In order to evaluate the contribution of the molecular size, dermatan sulfate samples (Nos. 8–11 in Table I) of widely different molecular weight but having similar C/O ratios were analyzed by the CPC–cellulose microcolumn technique at acid pH. It can be seen from Fig. 6 that their elution profiles differed only slightly with respect to the apparent CEC.

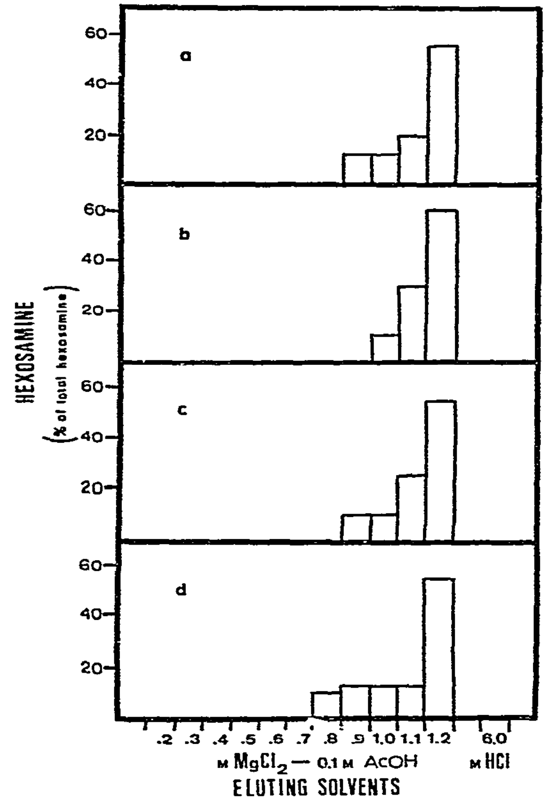

Fig. 6. CPC–cellulose microcolumn elution profiles ("acid magnesium chloride elution profiles") of (a) and (b) hyaluronidase-treated pig-skin dermatan sulfate (samples 10 and 11 in Table I, respectively), (c) pig-skin dermatan sulfate (sample 9), and (d) hyaluronidase-treated mucosa dermatan sulfate (sample 8).

Finally, dermatan sulfate samples having extremely variable molar ratios of sulfur to hexosamine (Nos. 3, 11, 13, 14 in Table I) were also examined by this technique at acid pH. It is seen from Fig. 7 that the degree of sulfation affected the

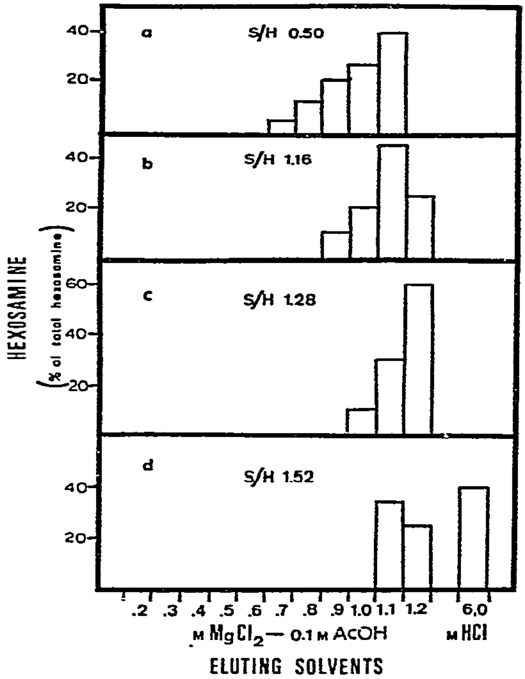

Fig. 7. CPC–cellulose microcolumn elution profiles ("acid magnesium chloride elution profiles") of (a) beef-aorta dermatan sulfate (sample 3 in Table I); (b), (c), and (d) hyaluronidase-treated pig-skin dermatan sulfates (samples 13, 11, and 14, respectively). S/H, molar ratio of sulfur to hexosamine.

elution profiles in the sense that the apparent CEC was displaced towards higher values with increasing molar ratios of sulfur to hexosamine. However, it may be calculated that almost a 30%-increase in sulfur content is required to increase the apparent CEC by 0.1M. On the other hand, an increase as small as 5% in the content of D-glucuronic acid causes a decrease in the apparent CEC by 0.1M (Fig. 5). Thus, the ratio of L-iduronic to D-glucuronic acid appears to be the major factor in determining the solubility characteristics at low pH.

Profiles of elution of cellulose with barium acetate–ethanol. — Since CPC–cellulose microcolumn elution profiles performed at acid pH reflected both the uronic acid composition as well as the sulfur content of the polysaccharide, it was of interest to see whether other techniques might provide means of assessing only one of these parameters.

As shown in Fig. 8, dermatan sulfate samples of varying uronic acid composition, as reflected by their C/O ratios, were subjected to barium acetate–ethanol cellulose fractionation. In contrast to the "acid magnesium chloride elution profiles", this technique separated polysaccharides having C/O ratios ranging from 0.24 to 2.14. Thus, even dermatan sulfates containing almost 50% of total uronic acid as D-glucuronic acid could be distinguished from chondroitin sulfate (Fig. 8,d and e).

The effect of molecular size as well as of sulfur content on the elution profiles in the barium acetate–ethanol system was also studied. As depicted in Fig. 9, dermatan sulfate samples of varying molecular weight showed no tendency to separate according to molecular size; instead, the material having the lowest C/O ratio (Fig. 9a) displayed the lowest solubility in ethanol. Finally, dermatan sulfate samples having varying

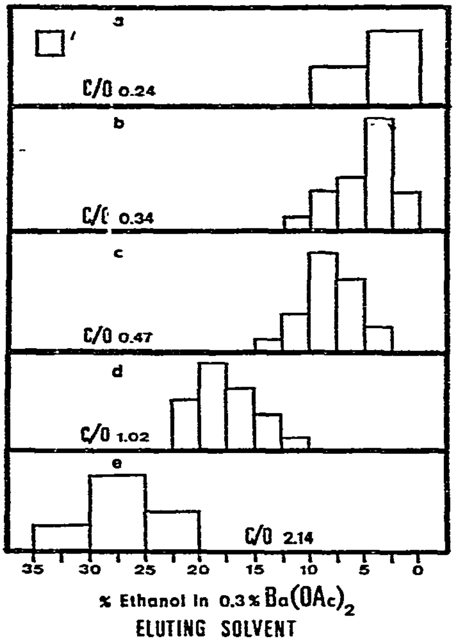

Fig. 8. Profiles of elution with barium acetate–ethanol of cellulose microcolumns: (a) Beef-aorta dermatan sulfate (Fraction III in Table II), (b) pig-skin dermatan sulfate (sample 9 in Table I), (c) and (d) horse-aorta dermatan sulfates (samples 15 and 17, respectively), and (e) chondroitin 4-sulfate. The values obtained are shown in the graphs with the area representing the hexosamine content (the reference square in the upper left corner equals 10% of the total hexosamine content). C/O, carbazole to orcinol ratio.

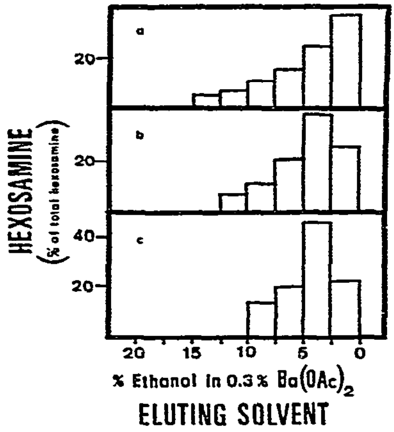

Fig. 9. Profiles of elution with barium acetate–ethanol of cellulose microcolumns: (a) and (b) pig-skin dermatan sulfates (samples 13 and 9, respectively, in Table I), and (c) sclera dermatan sulfate (sample 1).

molar ratios of sulfur to hexosamine were also examined by this technique. The profiles in Fig. 10 clearly demonstrate that, under these conditions, charge density had no effect on the elution profile, or, rather, the position of the peak of the elution profile. Again, the differences observed were solely due to variations in the C/O ratios (the samples shown in Fig. 10, a and b, had C/O ratios of 0.50 and 0.47, respectively, whereas the other samples all had C/O ratios of 0.29). Thus, barium acetate–ethanol cellulose elution profiles seem to reflect only the uronic acid composition of the material analyzed.

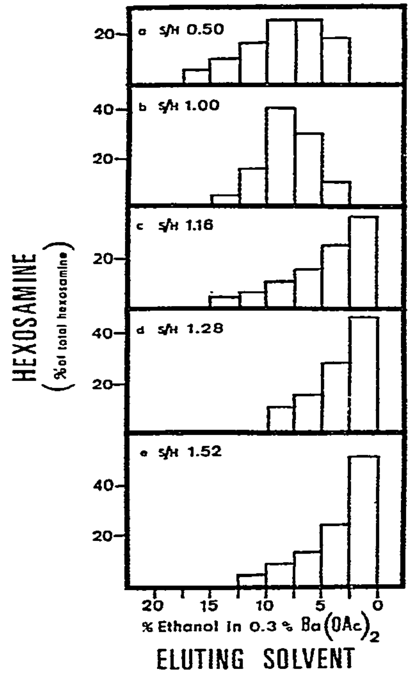

Fig. 10. Profiles of elution with barium acetate–ethanol of cellulose microcolumns: (a) Beef-aorta dermatan sulfate (sample 3 in Table I); (b) horse-aorta dermatan sulfate (sample 15); (c), (d), and (e) pig-skin dermatan sulfates (samples 13, 11, 14, respectively). S/H, molar ratio of sulfur to hexosamine.

Analyses of unknown dermatan sulfate samples. — The results presented thus far indicate that CPC–cellulose and barium acetate–ethanol cellulose microcolumn analyses can be utilized to study the hybrid nature of dermatan sulfates. Whereas the "neutral magnesium chloride elution profiles" reflect the molecular size polydispersity of the material, the relative uronic acid composition as well as variations in the sulfur content can be assessed by the "acid magnesium chloride elution profiles". Finally, barium acetate–ethanol cellulose elution profiles record exclusively the uronic acid composition of the polymer.

This approach was used to study the properties of hybrid dermatan sulfates from beef sclera and aorta, and from human knee-joint capsule. Before discussing the results obtained, a brief description of four hypothetical hybrid dermatan sulfate

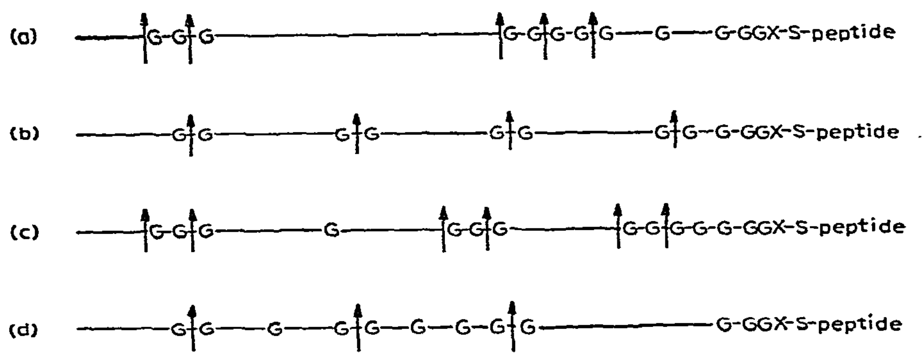

Fig. 11. Schematic models of some possible hybrid structures of dermatan sulfate. The straight line corresponds to sections of the molecule having L-iduronic acid-containing disaccharide units. D-Glucuronic acid-containing repeating periods are designated G. The arrows indicate linkages that are cleaved by testicular hyaluronidase. GGX indicates the neutral trisaccharide galactosylgalactosylxylose linked to the protein moiety. S, serine residue of the peptide moiety.

molecules will be presented (Fig. 11). The models differ in two principal ways: firstly, with regard to the length of the glucuronic acid-containing sections (called G) and, secondly, with regard to the distribution of these sections along the chains. Whereas model (a) contains G sections of varying length with an uneven distribution, model (b) contains G sections of equal length with equal spacing. Treatment of such hybrids with testicular hyaluronidase leads to the cleavage of the 2-acetamido-2-deoxy-β-D-glycopyranosyl linkages to the D-glucuronic acid units[1,2] resulting in a partial degradation of the molecule. (It should be pointed out that the linkages to single D-glucuronic acid residues do not seem to be susceptible to testicular hyaluronidase[2].) Thus, model (a) yields fragments of rather varying size which would be recorded in the "neutral magnesium chloride elution profile" as an increase in heterogeneity, *i.e.* the material would be eluted over a range of concentrations wider than that of the undegraded polymer. In addition, a decrease in the apparent CEC would also be observed. Furthermore, owing to the rather uneven distribution of D-glucuronic acid residues in these fragments, the "acid magnesium chloride elution profile" might be expected to be quite heterogeneous as well.

On the other hand, model (b) would yield fragments of rather equal size and equal content of D-glucuronic acid resulting in a limited heterogeneity in both the "neutral" and the "acid magnesium chloride elution profiles".

Model (c) contains G sections of varying length with an uneven distribution like model (a), with the exception that single G units are inserted into extended L-iduronic acid-containing sections. This results in the ratio between the uronic acids being similar in the short as well as in the long fragments. Thus, hyaluronidase degradation of this model is expected to yield a heterogeneous pattern in the "neutral magnesium chloride elution profile", whereas the "acid magnesium chloride elution profile" would show *no* increase in heterogeneity.

Model (d), finally, may be viewed as an extension of model (b) with insertion of single G units at varying frequency in the predominantly L-iduronic acid-containing sections of the molecule. Hyaluronidase treatment of this polymer would yield fragments of rather equal size but having considerable variation in their uronic acid composition. This would be observed as a relatively homogeneous "neutral magnesium chloride elution profile" and an extremely heterogeneous "acid magnesium chloride elution profile".

With these considerations in mind the data obtained for the three dermatan sulfate samples will be discussed.

(*a*) *Dermatan sulfate of beef sclera.* — This polysaccharide has a relatively high molecular weight (K_{av}, 0.19; CEC, 0.61) and contains approximately 10% of the total uronic acid content as D-glucuronic acid[25]. Treatment with testicular hyaluronidase resulted in a considerable depolymerization as indicated by the solubility curves (Fig. 1,a and b) and by the gel-chromatography pattern, which also demonstrated a marked molecular weight polydispersity (Fig. 3b). Although the fragments apparently varied greatly in size, their ratios of L-iduronic to D-glucuronic acid did not seem to differ to any large extent as indicated by the "acid magnesium chloride elution profiles" shown in Fig. 12, a and b. Therefore, the hybrid nature of beef-sclera dermatan sulfate can be described by model (c) of Fig. 11.

It should also be pointed out that the hyaluronidase-degraded material exhibited

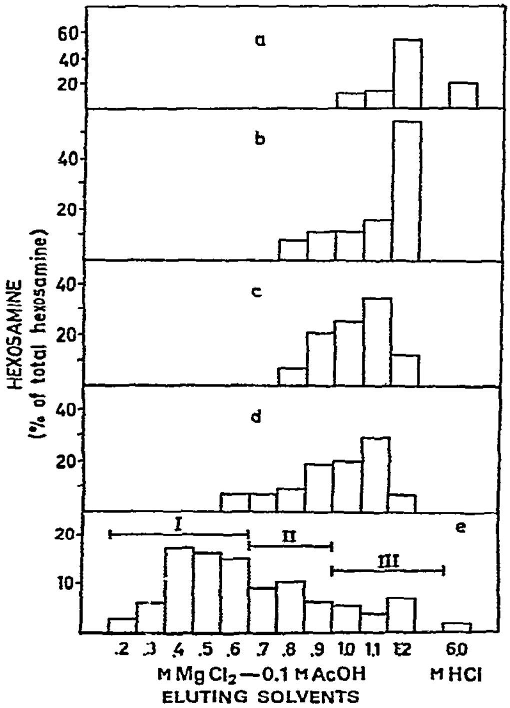

Fig. 12. CPC-cellulose microcolumn elution profiles ("acid magnesium chloride elution profiles") of beef-sclera dermatan sulfate (a) before and (b) after hyaluronidase treatment (samples 1 and 2 in Table I), joint-capsule dermatan sulfate (c) before and (d) after enzyme degradation (samples 5 and 6), and (e) hyaluronidase-treated beef-aorta dermatan sulfate (sample 4 in Table I).

a lower C/O ratio than the starting material (Table I) indicating that the polymeric fragments contained fewer G units than the intact polymer. This finding is also in agreement with model (c); the presence of several adjacent G units leads to a liberation of small fragments of oligosaccharide nature containing primarily D-glucuronic acid upon treatment with hyaluronidase. Infrared data (Table III) suggest that beef-sclera dermatan sulfate is composed primarily of 4-sulfated units. The possible presence of some 6-sulfated D-glucuronic acid-containing repeating units is suggested by the finding that the oligosaccharide split-products exhibited a slightly higher ratio of absorbancies at 800 and 850 cm^{-1}.

(*b*) *Dermatan sulfate of human knee-joint capsule.* — This dermatan sulfate had approximately 5–10% of the total uronic acid content as D-glucuronic acid (Table I). It was depolymerized by hyaluronidase (Fig. 1, g and h) to give fragments of reasonable uniformity (Fig. 3, c and d). Since the "acid magnesium chloride elution profile" (Fig. 12, c and d) showed little or no change after enzymic treatment, it is proposed that the molecule of joint-capsule dermatan sulfate rather closely resembles model (b) of Fig. 11. Furthermore, this dermatan sulfate showed almost identical C/O ratios before and after hyaluronidase treatment (Table I). These data are also in accordance with model (b). Although hyaluronidase-treated sclera and joint-capsule dermatan sulfates showed similar C/O ratios (Table I), the apparent CEC of the latter material was lower by approximately 0.04M than that of the former material. This is to be expected since joint-capsule dermatan sulfate was considerably "undersulfated" in comparison to the sclera material. The i.r. data in Table III do not suggest any specific distribution of the few 6-sulfated hexosamine units present in this polymer.

It is of interest that dermatan sulfate of human osteoarthritic knee-joint capsule, which was studied by Heinegård *et al.*[26], did not show any noticeable change of the neutral magnesium chloride elution profile after hyaluronidase treatment. However, the acid magnesium chloride elution profile demonstrated that a considerable proportion of the material exhibited an increased CEC after enzymic treatment. The data presented in this paper suggest that most, if not all, of the D-glucuronic acid-containing disaccharide units are located at the extremities of the polymer obtained from the diseased tissue. In contrast, the material from the normal tissue contains, in addition, internal hyaluronidase-susceptible bonds. Although the process leading to osteoarthritis may be accompanied by the *de novo* synthesis of a different dermatan sulfate, it is also conceivable that in this process dermatan sulfate may be transformed, *in situ*, *via* a chemical modification, *e.g.* by incorporation of sulfate groups into internal D-glucuronic acid residues. The presence of additional sulfate groups on uronic acid residues of dermatan sulfate has been demonstrated by Suzuki and coworkers[27]. Since the oversulfated disaccharides were obtained from a chondroitinase digest, it was not possible to ascertain whether the additional sulfate groups were located on the L-iduronic or D-glucuronic acid residues. However, assuming that the additional sulfate groups are located on the D-glucuronic acid residues, it is conceivable that 2-acetamido-2-deoxy-β-D-glycopyranosyl linkages to substituted glucuronic acid residues are resistant to the action of testicular hyaluronidase. On the other hand, hyaluronidase degradation of a polymer having an extremely uneven distribution of sulfate, and of sulfated uronic acid residues in particular, might yield fragments of rather varying charge density. Some of the fragments may even exhibit higher charge density than does the intact polymer. This could be an alternative explanation of the findings of Heinegård *et al.*[26].

(*c*) *Dermatan sulfate of beef-aorta.* — This polymer is characterized by a comparatively high D-glucuronic acid content (approximately 15% of the total uronic acid content) and shows a fairly low molecular weight, in the order of 25,000 (CEC, 0.47). The "neutral" elution profile obtained after hyaluronidase treatment indicated that the polysaccharide was degraded, on an average, to approximately half of its original size (CEC, 0.36). Since the "neutral" elution profile showed little increase in molecular-weight polydispersity (Fig. 13), it would appear that the hyaluronidase-susceptible regions of aorta dermatan sulfate are rather uniformly spaced along the chain. However, the hyaluronidase-degraded material displayed a considerable heterogeneity in the "acid magnesium chloride elution profile" (Fig. 12e) as compared to undegraded beef-aorta dermatan sulfate (Fig. 7a). Since "acid magnesium chloride profiles" record both variations in the uronic acid composition and variations in the sulfate content of the material, various fractions of beef-aorta dermatan sulfate obtained by CPC–cellulose fractionation of a hyaluronidase digest (Fig. 12e) were analyzed for uronic acid composition and sulfate content. As shown in Table II, the three fractions (I–III) differed with respect to their C/O ratios, whereas the sulfate-to-hexosamine molar ratio was little changed. These data seem to fit model (d) of Fig. 11. The observation that Fraction III has a molecular size slightly smaller than that of Fraction II (Fig. 3, e and f) further suggests that the fragments containing predominantly L-iduronic acid were generally shorter than the fragments having a large proportion of D-glucuronic acid residues. Thus, beef-aorta dermatan sulfate seems to be similar to pig-skin dermatan sulfate[1,2], both in terms of the distribution of the D-glucuronic acid residues and with respect to the location of the sulfate groups at the 4-position of the hexosamine moieties (Table III).

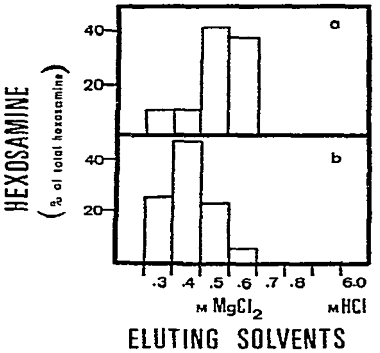

Fig. 13. CPC–cellulose microcolumn elution profiles ("neutral magnesium chloride elution profiles") of beef-aorta dermatan sulfate (a) before and (b) after hyaluronidase treatment (samples 3 and 4 in Table I, respectively).

TABLE III

INFRARED DATA OF VARIOUS DERMATAN SULFATE PREPARATIONS AND THEIR DEGRADATION PRODUCTS

Source	*Treatment*	*Absorption at 800 cm^{-1}* / *Absorption at 850 cm^{-1}*
Sclera	—	0.84
Sclera	H-sup[a]	0.98
Joint capsule	—	0.96
Joint capsule	H-sup[a]	1.00
Joint capsule	H-ppt[a]	0.94
Beef aorta	—	0.72

[a] H, Hyaluronidase treated sample; the dermatan sulfate samples were treated with testicular hyaluronidase and the polysaccharide material (H-ppt) was reisolated as previously described[1]. The supernatant solutions (H-sup) of the 50%-ethanol precipitation containing the oligosaccharide split products were desalted by passage through a column (1 × 90 cm) of Sephadex G-25 (fine) and elution with 10% ethanol.

It should be stressed that the various models in Fig. 11 are only schematic models showing some principal differences in the structure of various dermatan sulfates. The exact location of single G units as well as of clusters of such units can only be determined when a technique for sequence analysis of dermatan sulfates is available. However, microcolumn analyses in the manner described in this paper have been performed on dermatan sulfates isolated from corneal stroma in various stages of scar-tissue formation[28]. On the basis of the data obtained in the present study, it was concluded that the dermatan sulfate exhibited a higher degree of hybridity in the early stages of scar-tissue formation.

In conclusion, it is hoped that the approach utilized in the present investigation will be of value for studies of dermatan sulfates obtained from other tissues, normal as well as pathological. The present approach ought to be particularly useful in the analysis of specimens of limited availability as well as for the analysis of tissue sections.

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REFERENCES

1 L.-Å. Fransson and L. Rodén, *J. Biol. Chem.*, 242 (1967) 4161.
2 L.-Å. Fransson and L. Rodén, *J. Biol. Chem.*, 242 (1967) 4170.
3 L.-Å. Fransson, *J. Biol. Chem.*, 243 (1968) 1504.
4 L.-Å. Fransson, *Ark. Kemi*, 29 (1968) 95.
5 C. A. Antonopoulos, S. Gardell, J. A. Szirmai, and E. R. De Tyssonsk, *Biochim. Biophys. Acta*, 83 (1964) 1.
6 S. Thunell, *Ark. Kemi*, 27 (1967) 33.
7 C. A. Antonopoulos, E. Borelius, S. Gardell, B. Hammström, and J. E. Scott, *Biochim. Biophys. Acta*, 54 (1961) 213.

8 J. R. KIMMEL AND E. L. SMITH, *J. Biol. Chem.*, 207 (1954) 515.
9 L.-Å. FRANSSON, *Biochim. Biophys. Acta*, 156 (1968) 311.
10 L. A. ELSON AND W. T. J. MORGAN, *Biochem. J.*, 27 (1933) 1824.
11 Z. DISCHE, *J. Biol. Chem.*, 167 (1947) 189.
12 A. H. BROWN, *Arch. Biochem.* [*Biophys.*], 11 (1946) 269.
13 C. A. ANTONOPOULOS, *Acta Chem. Scand.*, 16 (1962) 1521.
14 T. C. LAURENT AND J. KILLANDER, *J. Chromatog.*, 14 (1964) 317.
15 C. A. ANTONOPOULOS AND S. GARDELL, *Acta Chem. Scand.*, 17 (1963) 1474.
16 S. GARDELL, *Acta Chem. Scand.*, 11 (1957) 668.
17 M. B. MATHEWS, *Arch. Biochem. Biophys.*, 61 (1956) 367.
18 M. B. MATHEWS AND A. DORFMAN, *Arch. Biochem. Biophys.*, 42 (1953) 41.
19 T. C. LAURENT AND J. E. SCOTT, *Nature*, 202 (1964) 661.
20 J. A. SZIRMAI, E. VAN BOVEN-DE TYSSONSK, AND S. GARDELL, *Biochim. Biophys. Acta*, 136 (1967) 331.
21 T. C. LAURENT, *Arch. Biochem. Biophys.*, 92 (1961) 224.
22 M. B. MATHEWS, *Biochim. Biophys. Acta*, 35 (1959) 9.
23 A. HAUG AND B. LARSEN, *Acta Chem. Scand.*, 15 (1961) 1395.
24 M. B. MATHEWS, *Biochim. Biophys. Acta*, 48 (1961) 402.
25 L.-Å. FRANSSON, L. RODÉN, AND M. L. SPACH, *Anal. Biochem.*, 23 (1968) 317.
26 D. HEINEGÅRD, J. HERNBORG, AND B. J. LUNDBERG, *Arthr. Rheum.*, 11 (1968) 787.
27 S. SUZUKI, H. SAITO, T. YAMAGATA, K. ANNO, N. SENO, Y. KAWAI, AND T. FURUHASHI, *J. Biol. Chem.*, 243 (1968) 1543.
28 A. ANSETH AND L.-Å. FRANSSON, *Exp. Eye Res.*, 8 (1969) 302.