Carbohydrate Research, 140 (1985) 239–249
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

CHROMATOGRAPHY OF GLYCOSAMINOGLYCANS ON HYDROPHOBIC GEL. CORRELATION BETWEEN CHROMATOGRAPHIC BEHAVIOR OF GLYCOSAMINOGLYCANS ON PHENYL-SEPHAROSE CL-4B AND THEIR SOLUBILITY IN THE PRESENCE OF HIGH CONCENTRATIONS OF AMMONIUM SULFATE

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(Received April 26th, 1983; accepted for publication revised form, December 12th, 1984)

ABSTRACT

The effect of bound sulfate groups and uronic acid residues of glycosaminoglycans on their behavior in chromatography on hydrophobic gel was examined by the use of several pairs of depolymerized chondroitin, chondroitin 4- or 6-sulfate, and dermatan sulfate having comparable degree of polymerization. Chromatography on Phenyl-Sepharose CL-4B in 4.0–2.0M ammonium sulfate containing 10mM hydrochloric acid showed that: (a) The retention of depolymerized chondroitin 4- or 6-sulfate on the gel varies with the temperature, whereas the depolymerized samples of chondroitin and dermatan sulfate does not show a temperature dependence (this is not the case for hyaluronic acid or dextrans). (b) Among depolymerized samples of chondroitin and chondroitin 4- and 6-sulfate that have a similar degree of polymerization, chondroitin 4- and 6-sulfate showed the highest retention. (c) The retention on the gel of chondroitin 6-sulfate, chondroitin 4-sulfate, and dermatan sulfate decreased in this order. The solubility in ammonium sulfate solution of the polysaccharides agreed well with the chromatographic behavior, suggesting that the fractionation by the hydrophobic gel largely depends on the ability to precipitate on the gel rather than on the hydrophobic interaction between gel and polysaccharide.

INTRODUCTION

Nagasawa and assoc.[1–3] reported that chromatography of glycosaminoglycans on hydrophobic gel in the presence of high concentration of ammonium sulfate in 10mM hydrochloric acid depends on the proportion of *N*-acetyl groups and the molecular size. Recently, the same authors[4] suggested that the fractionation of heparin on Sepharose CL-4B may depend on the solubility of the polysaccharide in

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0008-6215/85/$ 03.30

ammonium sulfate solutions, as well as on the hydrophobic interaction between gel and polysaccharide molecule. The present report describes results which indicate that the bound sulfate groups and uronic acid residues in glycosaminoglycans strongly affect the solubility properties in ammonium sulfate solution, which results in the different chromatographic behaviors on hydrophobic gels.

EXPERIMENTAL

Materials. — Chondroitin 6- and 4-sulfate, and hyaluronic acid (rooster-comb) were obtained from Seikagaku Kogyo Co., Ltd., Tokyo. Dextrans (M_r 11 000, 49 000, and 200 000) were supplied by Meito Sangyo Co., Nagoya. Depolymerized chondroitin fractions having various degrees of polymerization were those reported previously[2]. Standard of *N*-acetylchondrosine was prepared by acetylation of chondrosine (Seikagaku Kogyo Co.) according to the method of Danishefsky *et al.*[5]. Bovine testis hyaluronidase (Type IV, 1090 units/mg) and bovine liver β-D-glucuronidase (Type B-10, 12 000 units/mg) were obtained from Sigma Chemical Co., St. Louis, MO 63178. Phenyl-Sepharose CL-4B, Sepharose 6B, Sephadex G-25, G-75, and G-100 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Analytical methods. — The uronic acid content was determined by the modified method of Bitter and Muir[6], reducing 2-acetamido-2-deoxy-D-galactose content by the method of Reissig *et al.*[7], and sulfate content by a turbidimetric method[8]. The hexose content was determined by the phenol–H_2SO_4 method[9]. Analytical gel-chromatography on Sephadex G-75 was carried out by the the same procedure described previously[2], and the chromatography on Sepharose 6B was carried out by the aforementioned procedure, modified by the use of 0.2M NaCl as eluent and by changing the flow rate to 18 mL/h.

Preparation of depolymerized chondroitin 6- and 4-sulfate fractions having various degrees of polymerization. — A solution of the Na salt of chondroitin 6- or 4-sulfate (1 g) in 0.1M Na acetate–0.15M NaCl (pH 5.0, 665 mL) was incubated with a solution of bovine testis hyaluronidase (33.6 mg) in the same solvent (65 mL) for 2 h at 37° for chondroitin 6-sulfate, and for 1.5 h at 37° for chondroitin 4-sulfate, then heated in a boiling water-bath for 10 min. After dialysis against distilled water (10 L) overnight, the solution was concentrated *in vacuo* at 50° to ~10 mL, applied to a column (2.6 × 90 cm) of Sephadex G-100 prepared in 0.5M $(NH_4)_2CO_3$, pH 8.5, and eluted with the same solution at a flow rate of 10 mL/h at 20–25°. The eluate was collected in 5-mL fractions, and each fraction was analyzed for uronic acid content. The material eluted was pooled to give ten main fractions of equal volume, which were lyophilized. Each of the residues was dissolved in 0.5M $(NH_4)_2CO_3$ (5 mL) and the solution applied to a Sephadex G-75 or G-100 column (2.6 × 90 cm). The elution was monitored by the uronic acid assay. Rechromatography of the pooled samples was performed to give elution diagrams having sharp peaks for each fraction. Each residue from the rechromatography was dissolved in

a small volume of water and passed through a column (1 × 6.5 cm) of Dowex 50W (Na^+, 50–100 mesh), which was eluted with water. Each eluate was collected and lyophilized to give the Na salt of the product as a white powder. The preparations obtained were subjected to chemical analyses and analytical gel-chromatography on Sephadex G-75 gel, and were used for chromatography on Phenyl-Sepharose CL-4B (Table I and Fig. 2).

Preparation of a set of depolymerized chondroitin, and chondroitin 6- and 4-sulfate fractions having matched molecular-size distribution. — Chondroitin fraction. Desulfation–depolymerization of the pyridinium salt of chondroitin 6-sulfate (200 mg) in dimethyl sulfoxide containing 10% of water for 70 min at 90° was carried out by the procedure previously reported[2]. The depolymerized chondroitin (138 mg, Na salt) dissolved in 0.2M NaCl (5 mL) was applied to a column (2.5 × 84 cm) of Sepharose 6B which was eluted at room temperature with the same solution at a flow rate of 39 mL/h. The eluate was collected in 5.6-mL fractions, and each fraction was analyzed for uronic acid content. The fractions (fraction number 50–57) were pooled, dialyzed, and freeze-dried (41 mg).

Chondroitin 6-sulfate fraction. The Na salt of chondroitin 6-sulfate (200 mg), dissolved in 0.1M Na acetate–0.15M NaCl (pH 5.0, 5 mL), was digested with bovine testis hyaluronidase (1.0 mg) for 1 h at 37°. The mixture was applied to a column (2.5 × 84 cm) of Sepharose 6B prepared in 0.2M NaCl, which was eluted as described earlier. The corresponding fractions (number 50–57) were pooled, dialyzed, and freeze-dried (59 mg).

Chondroitin 4-sulfate fraction. The Na salt of chondroitin 4-sulfate (200 mg) was fractionated on a Sepharose 6B column by the procedure described earlier. The pooled fractions were dialyzed and freeze-dried (59 mg).

Rechromatography of the glycosaminoglycan fractions on Sepharose 6B was performed under identical conditions. Analytical gel-chromatography of the purified materials on Sepharose 6B gave an elution diagram with a sharp peak and nearly identical molecular-size distribution for each glycosaminoglycan (K_{av} 0.52, elution diagrams not shown). The solubility in ammonium sulfate solution of the materials thus obtained was determined (Fig. 4a).

Preparation of a pair of dermatan sulfate and chondroitin 4-sulfate fractions having matched molecular-size distribution. — Dermatan sulfate fraction. The chondroitinase AC-II-resistant fraction (18.9 mg, sodium salt) was obtained from rooster-comb dermatan sulfate RC-20 (40 mg) by the method previously reported[13]. This fraction, which was considered to consist of highly polymerized *N*-acetyldermosine 4-sulfate, was chromatographed on a Sepharose 6B column (2.5 × 85 cm) prepared in 0.2M NaCl at 20°. The column was eluted with the same solution at a flow rate of 39 mL/h. The eluate was collected in 5-mL fractions, and each fraction was analyzed for uronic acid content. Fractions number 47–53 were pooled, dialyzed, and freeze-dried (16.1 mg).

Chondroitin 4-sulfate fraction. Fractionation of chondroitin 4-sulfate (sodium salt, 200 mg) on Sepharose 6B gel under conditions identical with those used for the dermatan sulfate yielded a white powder (16.1 mg).

Analytical gel-chromatography of these fractions on Sepharose 6B gave an elution diagram with a sharp peak and nearly identical molecular-size distribution for each fraction (K_{av} 0.35, elution diagrams not shown). They were used for chromatography on Phenyl-Sepharose CL-4B and the solubility in ammonium sulfate solution was determined (Fig. 3 and 4b).

Determination of the degree of polymerization. — The average degree of polymerization of depolymerized chondroitin 6- and 4-sulfate fractions was estimated by two different methods. One of the methods, which is essentially the same as described previously[2], is based on the determination of the molar ratio of reducing 2-acetamido-2-deoxy-D-galactose to uronic acid, as compared to that for a standard of *N*-acetylchondrosine. Another method, which has been described by several investigators[10–12], is based on the determination of the molar ratio of D-glucuronic acid at the nonreducing terminal to the total uronic acid as follows: A solution of the sample (2–4 mg) in 50mM acetate buffer (pH 5.0, 1 mL) was incubated with bovine liver β-D-glucuronidase (0.46–0.92 mg) for 3 h at 37°, and then heated in a boiling water-bath for 2 min. The mixture was applied to a column (1.6 × 93 cm) of Sephadex G-25, prepared in 0.1M NaCl, and eluted with the same solution at a flow rate of 20 mL/h at room temperature. The eluate was collected in 2-mL fractions, and each fraction was analyzed for uronic acid content. The amounts of D-glucuronic acid at the nonreducing terminal and of total uronic acid were determined from the peak areas corresponding to D-glucuronic acid and residual oligosaccharide of the elution diagram.

Chromatography of hyaluronic acid on Phenyl-Sepharose CL-4B. — A solution of the sample (~3 mg) in 3.0M $(NH_4)_2SO_4$ (pH 5.3, 3 mL) was applied to a column (1.0 × 20 cm) of Phenyl-Sepharose CL-4B prepared in the same solution. The column was eluted with a reverse linear-gradient (430 mL) of 3.0M $(NH_4)_2SO_4$–water at 25–27° or at 4°. The flow rate was 40 mL/h, and 7-mL fractions were collected. An aliquot (0.5 mL) was taken for the carbazole reaction and determination of the ionic strength.

Chromatography of dextrans having different molecular weights on Phenyl-Sepharose CL-4B. — A solution of the sample (dextran of M_r 11 000 or 49 000, ~5.1 mg) in 4.0M $(NH_4)_2SO_4$ in 10mM HCl (pH 3.4, 1 mL) was applied to a column (0.6 × 6 cm) of Phenyl-Sepharose CL-4B prepared in the same solution. The column was eluted with a reverse linear-gradient (100 mL) of 4.0–2.0M $(NH_4)_2SO_4$ in 10mM HCl at room temperature or at 4°. The flow rate was 15 mL/h and 2-mL fractions were collected. An aliquot (0.5 mL) was taken for the phenol–sulfuric acid reaction and determination of the ionic strength. In chromatography of dextran of M_r 200 000, the same column prepared in 3.4M $(NH_4)_2SO_4$ in 10mM HCl was used and eluted with a reverse linear gradient (100 mL) of 3.4–2.0M $(NH_4)_2SO_4$ in 10mM HCl.

Chromatography of depolymerized chondroitin, and chondroitin 6- and 4-sulfate fractions on Phenyl-Sepharose CL-4B. — Each fraction (Na salt, ~1.7 mg) was applied to a column (0.6 × 6 cm) of Phenyl-Sepharose CL-4B, prepared in

4.0M $(NH_4)_2SO_4$ in 10mM HCl, and eluted with a reverse linear-gradient at the indicated temperature, as described previously[2]. Each fraction was characterized by the concentration of $(NH_4)_2SO_4$ at the peak of elution (Table I and Fig. 2).

Chromatography of a pair of dermatan sulfate and chondroitin 4-sulfate fractions having matched molecular-size distribution. — A solution of the sample (~2 mg) in 2.8M $(NH_4)_2SO_4$ in 10mM HCl (pH 3.3, 2 mL) was applied to a column (0.6 × 6 cm) of Phenyl-Sepharose CL-4B prepared in the same solution. The column was eluted with a reverse linear-gradient (100 mL) of 2.8–1.0M $(NH_4)_2SO_4$ in 10mM HCl at 22–25° or at 4°. The flow rate was 30 mL/h and 2-mL fractions were collected. An aliquot (0.5 mL) was taken for the carbazole reaction and determination of the ionic strength (Fig. 3).

Determination of the solubility of a set of chondroitin, chondroitin 6- and 4-sulfate, and dermatan sulfate fractions having matched molecular-size distributions. — The sample (4.0 mg) was dissolved in 2.0M $(NH_4)_2SO_4$ in 10mM HCl. Aliquots (50 μL) of this solution were placed into Pyrex-glass centrifugation tubes (0.9 × 10 cm). To each tube were added 2.0M and 4.0M $(NH_4)_2SO_4$ in 10mM HCl to prepare samples (2.0 mL) having a 3.8–2.0M concentration of $(NH_4)_2SO_4$. Each sample was kept for 42 h at 4° or at 25°, and then centrifuged (3 000 r.p.m. 15 min) at 4° or at 25°. An aliquot (0.5 mL) of the supernatant solution was analyzed for uronic acid content. The solubility (%) of a sample at various concentrations of $(NH_4)_2SO_4$ in 10mM HCl was calculated from the ratio of the uronic acid contents under the conditions used *vs.* in 2.0M $(NH_4)_2SO_4$ in 10mM HCl.

RESULTS AND DISCUSSION

Previously it was reported that when heparin and chondroitin 6-sulfate were retained on hydrophobic gels, such as Phenyl-Sepharose CL-4B in the presence of high concentration of ammonium sulfate in 10mM hydrochloric acid, their retention on the gel increased as the temperature decreased[14]. Fig. 1 shows reverse linear-gradient elution diagrams of hyaluronic acid (M_r 2 000 000, determined by viscometry) in 3.0M ammonium sulfate–water on Phenyl-Sepharose CL-4B. Hyaluronic acid was completely retained on the gel in a 3.0M concentration of ammonium sulfate either at room temperature or at 4°, and the elution peak at 1.6M concentration was followed by a broad tailing. Similar behavior of non-sulfated, water-soluble glucans was demonstrated by the elution of dextrans having various mol.wt. (M_r 11 000, 49 000, and 200 000) on Phenyl-Sepharose CL-4B (4.0–3.0M ammonium sulfate in 10mM hydrochloric acid) at room temperature and at 4°. The retention of the water-soluble glucans on the gel was not affected by changing the temperature from 24.5–25.5° to 4° (data not shown). In a preliminary experiment, chondroitin devoid of sulfate ester groups was shown to differ from chondroitin 6-sulfate both in the retention on the gel as well as in temperature dependence.

As suggested in the preceding paper[4], it seems that the fractionation of

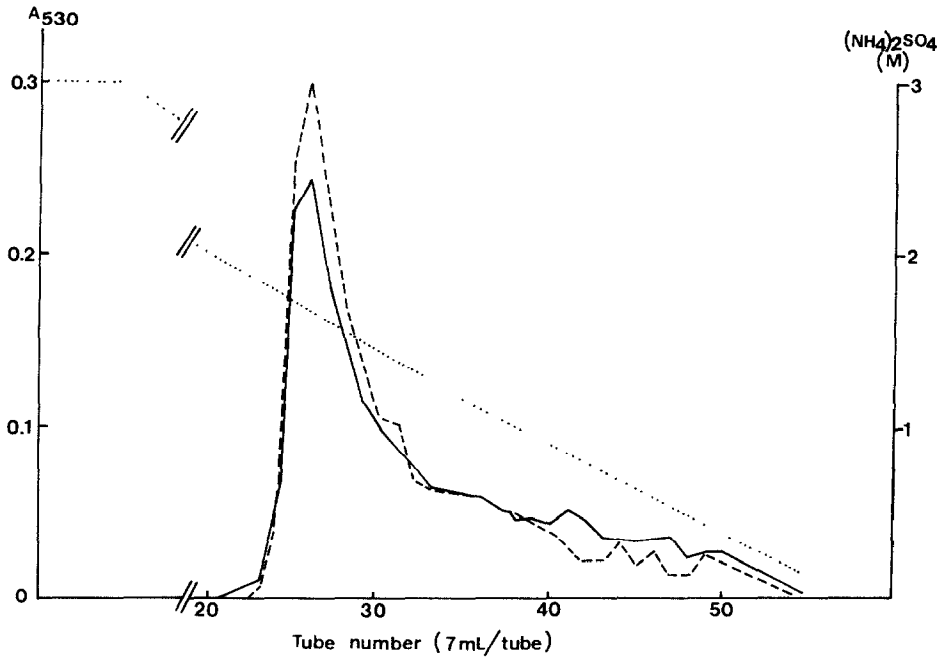

Fig. 1. Elution diagrams on Phenyl-Sepharose CL-4B, as determined by the carbazole reaction, of hyaluronic acid at 25–27° (——) and at 4° (------); (·····) concentration of ammonium sulfate solution.

glycosaminoglycans, such as heparin, on Phenyl-Sepharose CL-4B or highly cross-linked Sepharose 4B does not depend primarily on the hydrophobic interaction between the polysaccharide molecules and the gel, but depends predominantly on the solubility (or ability to precipitate) of the glycosaminoglycans in the presence of high concentrations of ammonium sulfate. Hence, the structural and physical properties that affect the retention of these polysaccharides on Phenyl-Sepharose CL-4B and its temperature dependency were investigated.

Depolymerized chondroitin and chondroitin sulfate fractions having various degrees of polymerization were prepared, and several pairs of depolymerized chondroitin, chondroitin 6-sulfate, chondroitin 4-sulfate, and dermatan sulfate fractions having comparable degrees of polymerization were investigated. The chemical and physical properties of these fractions are reported in Table I. The degree of polymerization was determined by use of the molar ratio of reducing 2-acetamido-2-deoxy-D-galactose to the total uronic acid content and the molar ratio of the D-glucuronic acid liberated by bovine liver β-D-glucuronidase digestion to the total uronic acid content. Both determinations were in agreement (data obtained by the former method are given in Table I). In order to save space, the elution patterns of each fraction obtained by gel-filtration are not given, but they are expressed as the ratio of the width of the base portion of each elution peak to the peak height (Table I). These data suggest that each fraction appears to be reasonably homogeneous with respect to molecular size.

TABLE I

ANALYTICAL DATA FOR SEVERAL PAIRS OF CHONDROITIN, CHONDROITIN 6- AND 4-SULFATE, AND DERMATAN SULFATE FRACTIONS HAVING MATCHED DEGREES OF POLYMERIZATION OR MOLECULAR-SIZE DISTRIBUTION

Glycosaminoglycan	*Uronic acid content (%)*	*S (%)*	*D.p.$_{av}$*[a]	K_{av} *on*		*Position of elution on Phenyl-Sepharose CL-4B (M ammonium sulfate)*[b]	
				Sephadex G-75	*Sepharose 6B*	*23–26°*	*4°*
Chondroitin	42.7[c]		26[c]	0.19(1:5.2)[d]		3.68	3.63
	44.6[c]		17[c]	0.34(1:5.0)		3.90	3.82
Chondroitin 6-sulfate	37.2	6.24	23	0.18(1:4.6)		2.68	2.36
	35.5	6.03	16	0.30(1:4.0)		3.05	2.68
Chondroitin 4-sulfate	37.9	6.11	23	0.16(1:4.4)		3.36	2.70
	35.4	6.11	16	0.28(1:4.6)		3.90	3.54
Dermatan sulfate					0.35	2.80	2.80
Chondroitin 4-sulfate					0.35	2.50	2.24

[a]See the Experimental section. [b]Expressed as the concentration of the ammonium sulfate eluent at the peak of elution (see Figs. 2 and 3). [c]Ref. 2. [d]In parentheses, ratio of width to height for each peak as an indication of the elution profile.

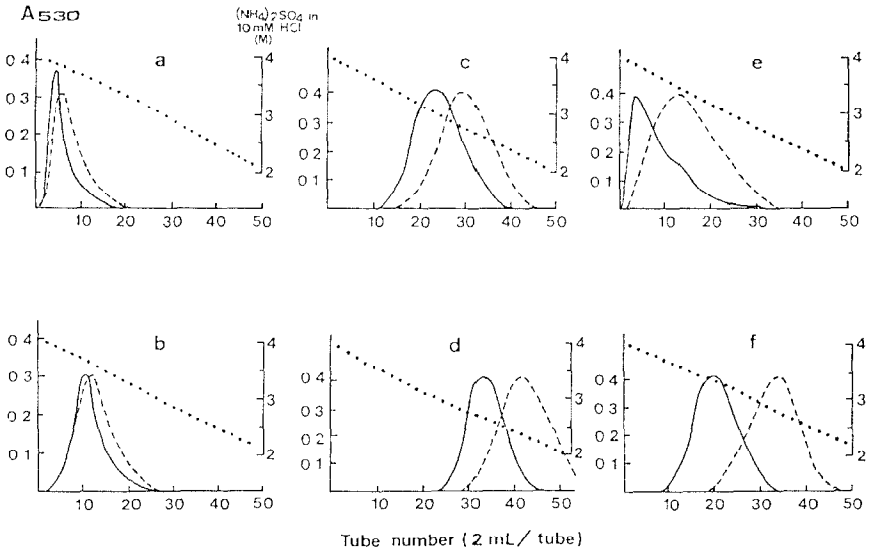

Fig. 2. Elution diagrams, on Phenyl-Sepharose CL-4B, of depolymerized chondroitin, and chondroitin 6- and 4-sulfate fractions having matched degrees of polymerization: Depolymerized chondroitin fraction (a, d.p. 17; b, d.p. 26), depolymerized chondroitin 6-sulfate fraction (c, d.p. 16, d, d.p. 23), and depolymerized chondroitin 4-sulfate fraction (e, d.p. 16; f, d.p. 23). Carbazole reaction· (——) at 23–26° and (------) at 4°; (·····) concentration of ammonium sulfate in 10mM hydrochloric acid

Elution diagrams, on Phenyl-Sepharose CL-4B, of pairs of depolymerized chondroitin, chondroitin 6-sulfate, and chondroitin 4-sulfate fractions having comparable degrees of polymerization are shown in Fig. 2. The position of peak elution for each fraction obtained from the gel with 4.0–2.0M ammonium sulfate in 10mM hydrochloric acid is expressed by the molar concentration of ammonium sulfate (Table I). Chondroitin 6- and 4-sulfate were more strongly retained on the gel than chondroitin at room temperature and at 4°. The results in Fig. 2 clearly indicate that the retention of these chondroitin sulfates on the gel increased as the temperature decreased. In contrast, chondroitin fractions exhibited very little increase in retention on the gel at the lower temperature. A similar behavior of other chondroitin fractions having d.p. 11 and 34 was also observed (data not shown). As shown also in Fig. 2, the chondroitin 6-sulfate fractions were more strongly retained on the gel than the corresponding fractions of chondroitin 4-sulfate at either temperature.

A pair of chondroitin 4-sulfate and dermatan sulfate fractions having substantially similar molecular-size distributions (K_{av} on Sepharose 6B: 0.35) was prepared in order to study the contribution of the uronic acid residue to the retention on the hydrophobic gel. As seen in Fig. 3, dermatan sulfate clearly shows less retention on the gel than did chondroitin 4-sulfate at either temperature, and the retention of the former was not affected by temperature changes. These results apparently indicate that the position of sulfate groups and the configuration of the uronic acid

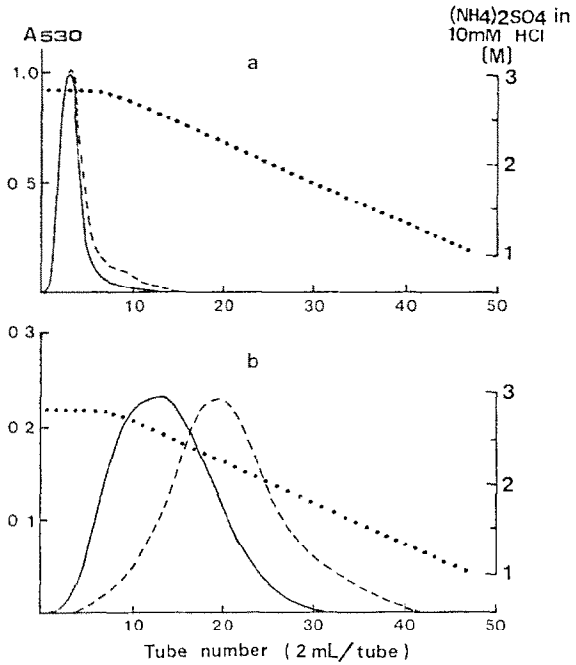

Fig. 3. Elution diagrams, on Phenyl-Sepharose CL-4B, of a pair of dermatan sulfate and chondroitin 4-sulfate fractions having matched molecular-size distributions: (a) Dermatan sulfate fraction (K_{av} on Sepharose 6B: 0.35), and (b) chondroitin 4-sulfate fraction (K_{av} on Sepharose 6B: 0.35). Carbazole reaction (——) at 22–25° and (------) at 4°; (·····) concentration of ammonium sulfate in 10mM hydrochloric acid.

residues in glycosaminoglycans affect the retention of the polysaccharides on the hydrophobic gel, and that the effect of the temperature on the retention on the gel does not necessarily depend on the presence of sulfate groups. Since the retention of glycosaminoglycans on an hydrophobic gel may be considered to depend mainly on the solubility in ammonium sulfate solution[4], it is possible that the structural differences among glycosaminoglycans do not affect directly the chromatographic behavior, but only indirectly through some physical properties, such as solubility. Thus, several pairs of glycosaminoglycan fractions having similar molecular-size distributions were prepared, and the solubility in ammonium sulfate solution determined at 25° and 4°. As shown in Fig. 4a, the solubilities of both chondroitin 6- and 4-sulfate fractions (each K_{av} on Sepharose 6B: 0.52) in ammonium sulfate solution were markedly lower than that of the chondroitin fraction (K_{av} on Sepharose 6B: 0.52) at either temperature, and the solubility dependence on the temperature was more distinct for the first two glycosaminoglycans than for the third. Comparison between the chondroitin 6- and 4-sulfate fractions showed that the former is much less soluble than the latter at either temperature. The solubility differences between chondroitin 4-sulfates of K_{av} 0.52 and K_{av} 0.35 (Figs. 4a,b) might be due to differences in their molecular weight, as observed on a series of chondroitin having different

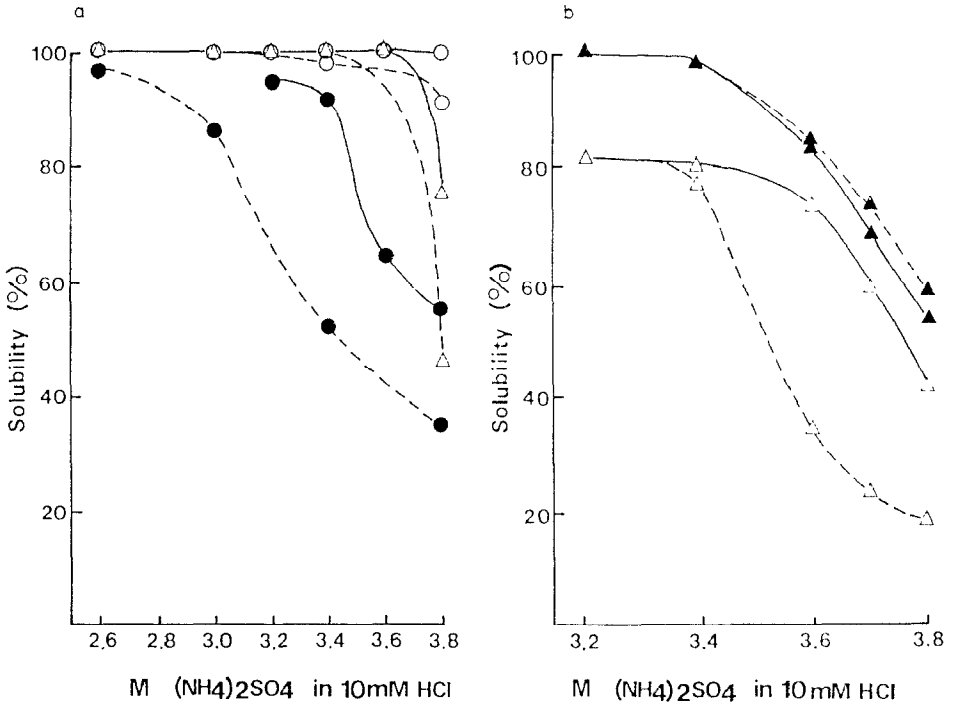

Fig. 4. (a) Solubility diagrams of a set of chondroitin, and chondroitin 6- and 4-sulfate fractions having matched molecular-size distributions, in ammonium sulfate in 10mM hydrochloric acid. (—○—) Chondroitin fraction (K_{av} on Sepharose 6B: 0.52), (—●—) chondroitin 6-sulfate fraction (K_{av} on Sepharose 6B: 0.52), and (—△—) chondroitin 4-sulfate fraction (K_{av} on Sepharose 6B: 0.52); (——) measured at 25° and (------) at 4°. (b) Solubility diagrams of a pair of dermatan sulfate and chondroitin 4-sulfate fractions having matched molecular-size distributions in ammonium sulfate in 10mM hydrochloric acid: (—▲—) Dermatan sulfate fraction (K_{av} on Sepharose 6B: 0.35) and (—△—) chondroitin 4-sulfate fraction (K_{av} on Sepharose 6B: 0.35); (——) measured at 25° and (------) at 4°.

degrees of polymerization (data not shown). The solubilities in ammonium sulfate solution of the dermatan sulfate fraction (K_{av} on Sepharose 6B: 0.35), obtained from the digestion products of rooster-comb dermatan sulfate (RC-20)[13] with chondroitinase AC-II, and of the chondroitin 4-sulfate fraction (K_{av} on Sepharose 6B: 0.35) were also studied. As seen in Fig. 4b, the solubility of the latter was lower than that of the former at either temperature, and the solubility of the former was not affected by the temperature. The reasons for the similarity in solubility between chondroitin (K_{av} 0.52) and chondroitin 4-sulfate (K_{av} 0.52) at concentrations of ammonium sulfate lower than 3.6M (Fig. 4a, at 25°) are not clear. Thus, the solubility properties of the glycosaminoglycan fractions tested were in good agreement with the chromatographic behaviors on a hydrophobic gel. Since the solubility of glycosaminoglycans is closely related to their ability to precipitate on the gel surface under the chromatographic conditions used, the correlation between solubility and retention strongly suggests that the fractionation of glycosamino-

glycans on hydrophobic gels largely depends on the mechanism proposed previously[4]. In the presence of high concentrations of ammonium sulfate, the structure of the water molecules surrounding the glycosaminoglycan molecules is greatly changed, resulting in a "pre-salting out" state. In this situation, the glycosaminoglycan molecules are retained by the gel through "interfacial precipitation" on the gel matrix and are released progressively from the gel as the salt concentration is reduced.

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