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Chromatography on ECTEOLA * of Sulfate Containing Mucopolysaccharides

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The chromatographic behaviour at different pH of hyaluronic acid, chondroitinsulfuric acid and heparin on ECTEOLA cellulose columns was studied. At acid pH good separations of the three polysaccharides from each other were obtained either by step-wise or gradient elution chromatography. Heparin preparations could be fractionated into subfractions of different anticoagulant activity. When the chromatography was carried out at +4°C no detectable inactivation of heparin occurred. The recovery of polysaccharides from the columns was close to 100 %.

A great number of different methods have been used for the preparation and fractionation of acid mucopolysaccharides (see *e.g.* Ref.[1-3]). Most of these methods have involved salt or alcohol fractionation, or electrophoresis. These methods are not easily adapted for a rapid preparation of small quantities of polysaccharides, as is often desired in tracer studies and enzyme experiments. An exception in this respect is the cetylpyridinium method of Scott [2,4,5].

Paper chromatography has not been used to any large extent, probably because of difficulties in finding a system where the polysaccharides give reproducible R_F values.

Ion exchange chromatography on Dowex 1 was used by Davidson and Meyer [6] for the preparation of chondroitin.

We have previously described briefly the use of the cellulose anion exchanger ECTEOLA for the fractionation of acid mucopolysaccharides [7]. This paper reports these experiments in full and describes some further extensions from them.

ECTEOLA was originally introduced by Peterson and Sober [8] for the chromatography of proteins. So far, however, it has been used mainly in the fractionation of nucleotides and polynucleotides [9,10].

* For brevity, this anion exchanger will be referred to here simply as ECTEOLA. This designation is derived from a contraction of the words epichlorohydrin and triethanolamine.

MATERIALS AND METHODS

Polysaccharides. Several batches of polysaccharides prepared according to different methods were used in this investigation *. However, the experiments shown in Tables 2—4 were all performed with the same polysaccharides. Some analytical data for the main polysaccharide preparations studied are shown in Table 1. It can be seen that hyaluronic acid was contaminated with materials containing galactosamine and sulfate. It is likely that this was due to the presence of a small quantity of chondroitin sulfuric acid.

Packing of columns. ECTEOLA cellulose (commercial grade, 0.39 meq/g) prepared according to Peterson and Sober [9], was obtained from Brown Company, Berlin, New Hampshire, USA. Before use, about 50 g of ECTEOLA cellulose was suspended in 0.5 M NaCl and packed in a large glass column. The column was then washed with 5 liters of 0.5 M NaOH, 5 liters of 3 M NaCl and 3 liters of 0.1 M NaCl—HCl (1:1). These washings were essential, otherwise materials giving various reactions for sugars were eluted in the chromatograms. The washed resin was then resuspended in 0.1 M NaCl—HCl (1:1) and packed into smaller columns for chromatography.

Polysaccharide solutions in water were adjusted to the pH used for chromatography by the addition of small quantities of the salt solution used for starting the chromatogram. The final solutions, containing 0.5—2 mg polysaccharide/ml with a salt concentration of less than 0.05 M, were allowed to drain into the column by gravity.

Elution of columns: Four different systems were used for elution of the columns:

A. 0.1—3.0 M HCl.
B. 0.1—3.0 M NaCl—HCl (1:1), pH 1.3 **.
C. 0.1—2.5 M NaCl in 0.05 M Glycine-HCl-buffer, pH 2.9 **.
D. 0.1—2.5 M NaCl in 0.05 M Sodium acetate buffer, pH 5.0 **.

Table 1. Analytical data †of polysaccharides used.

Polysaccharide	Source	Abbrev.	% amino sugar[15]	% amino glucose amine[16]	% of sugar as galactose amine[16]	% uronic acid[17]	% S[18,19]	% N	% Moisture	Ash %
Hyaluronic acid	Umbil. cord	HYA	38.2	90.4	9.6	36.6	0.6	3.56	14.5	8.0
Chondroitin sulfuric acid A	Calf cartilage	CHS-A	25.4	<3.0	100.0	27.7	4.3	2.58	12.0	24.5
Heparin Anticoag. 100 U/mg	Beef lung	HEP 100	24.5	100.0	0	20.8	10.4	2.18	13.7	39.4
Heparin by-product Polysaccharide II Anticoag. 5 U/mg	Beef lung	HBP II	12.9	51.5	48.5				9.1	
Heparin Anticoag. 120 U/mg	Beef lung	HEP 120	23.2						13.3	

* We wish to thank Drs. A. Balasz, S. Gardell, B. Högberg, T. Laurent, J. Scott and Vitrum Company for generous gifts of polysaccharide preparations.

** pH was measured on 0.1 M solutions.

† All figures for air dry preparations.

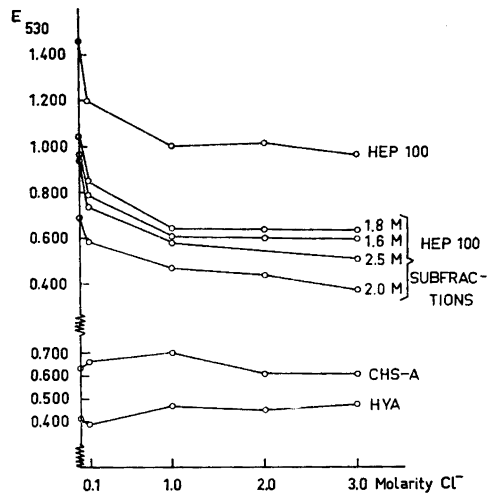

Fig. 1. Effect of different concentrations of HCl—NaCl (1:1) on the development of color in the carbazol reaction.

In a few exceptional cases when the polysaccharide was not completely eluted in these systems, the columns were at the end eluted with 0.5 M NaOH.

The columns were either eluted step-wise or by a gradient elution system which gave a linear increase in chloride concentration [11]. During step-wise elution a total of 100—150 ml of solution was used in each step with 1 × 10 cm ECTEOLA columns containing 10—15 mg of polysaccharide. The columns were eluted with the same eluent until the effluent contained less than 10 μg uronic acid/ml. In most cases 10—20 ml fractions were collected with an automatic fraction collector. The rate of elution during both gradient and step-wise chromatography was 10—20 ml/hour for a 10 × 1 cm column.

Analysis of fractions. The polysaccharides were localized in the different chromatographic fractions with the carbazol method [12]. For keratosulfate the anthrone reaction [13] was used. Salt concentration of the effluent was determined by conductivity measurements.

The influence of salt concentration on the results obtained by the carbazol method was studied in model experiments (Fig. 1). It is seen that, with heparin, increasing salt concentration (HCl—NaCl, 1:1) depressed the color development by about 40 %. No similar effect was observed for hyaluronic acid or chondroitin sulfuric acid. Instead, with hyaluronic acid a slight increase in color was observed at higher salt concentrations, while the effect on chondroitin sulfuric acid was negligable. Since high salt concentrations depressed the color development with heparin, it was considered necessary to investigate whether subfractions of heparin gave the same results. This was found to be the case (*cf.* Fig. 1), although minor quantitative differences could be observed.

The different heparin fractions were eluted at salt concentrations between 1.5 and 3.0 M Cl^- (system A), where no changes took place in the influence of salt on color development. Consequently no correction for the salt effect between different heparin fractions was necessary.

For heparin the carbazol reaction normally gives uronic acid values that are about 60 % higher than the theoretical ones [12]. The depression in color formation observed for heparin in the presence of chloride almost abolishes this anomaly and makes possible a more accurate quantitative interpretation of the carbazol reaction in the effluent. The errors in the quantitative estimation of chondroitin sulfuric acid and hyaluronic acid due to the presence of salt were found to be less than 8 %.

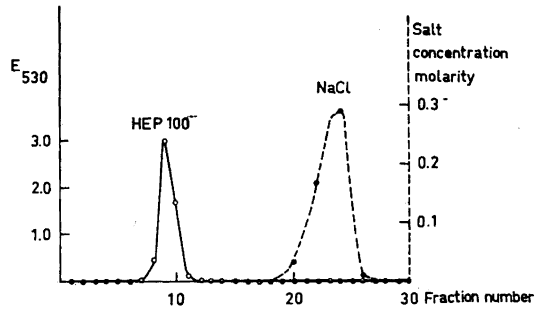

Fig. 2. Separation by gel-filtration on a Sephadex column (14 × 2 cm) of 2.5 mg of HEP 100 from 110 mg of NaCl.

Removal of salt. It was necessary to remove salts before more detailed analysis of the polysaccharide fractions could be made. This was done by dialysis at 0°C for 24 h against distilled water in tubing obtained from Visking Corporation, Chicago, Ill., USA. The solution was then neutralized to pH 7 with NaOH and evaporated under vacuum to a small volume. The polysaccharides were precipitated with 3—4 volumes of ethanol. The precipitate was then washed with 75 % ethanol, dried in a vacuum, weighed and analyzed.

When it was expected that an appreciable amount of polysaccharide would be lost during dialysis, a gel filtration technique was used [14]. This is illustrated by Fig. 2 which shows the separation of 2.5 mg of heparin (HEP 100) and 110 mg of NaCl by gel filtration on Sephadex G 25 (Pharmacia, Uppsala, Sweden). The materials were dissolved in 1 ml of water and introduced into the column by gravity. Elution was carried out with distilled water. Fractions were collected by a fraction collector (1.5 ml/30 sec). The polysaccharide could be completely separated from salt, the whole procedure being completed in less than 10 min.

Analytical methods. The total amino sugar content of the polysaccharides was determined by a modification of the Elson-Morgan procedure [15]. Glucosamine and galactosamine were separated and determined according to Gardell [16], uronic acid according to Tracey [17]. Sulfate was analyzed by the benzidine method [18] as modified by Balazs [19]. In this last modification the benzidine sulfate precipitate was collected on bacterial filters (Ra. Millipore Filter Comp., Bedford, Mass., USA.). Nitrogen was determined according to Kjeldahl. For moisture determinations the polysaccharides were dried under vacuum over P_2O_5 for 22 h at 90°C. Anticoagulant activity * was determined according to Studer and Winterstein [20].

RESULTS AND DISCUSSION

Tables 2, 3 and 4 give a survey of the chromatographic behaviour of hyaluronic acid (HYA), chondroitinssulfuric acid (CHS-A) and heparin (HEP 100) in four different chromatographic systems (*cf.* material and methods).

Hyaluronic acid (Table 2) was found to be very loosely or not at all bound to the ion exchanger in systems A and B. At the higher pH values of systems C and D it was adsorbed to the exchanger. In a gradient chromatogram with system D hyaluronic acid was eluted as a sharp peak between 0.4—0.5 M chloride.

* We wish to thank Prof. E. Jorpes, mrs Sohlberg and Vitrum Company for help with the determination of anticoagulant activities.

Table 2. Chromatographic behaviour of hyaluronic acid on ECTEOLA.

Fraction (Molarity Cl^-)	Amount of polysaccharide in % of total quantity eluted in different fractions	
	System B (and A)	System C (and D)
0.1	92	0
0.7	0	92
0.9	0	0
1.1	8	8

The calculations are based on the carbazol reaction applied directly to the eluted fractions.

The data in Tables 2—4 were obtained from chromatograms of 10—15 mg of polysaccharide on ECTEOLA columns, 1 × 10 cm.

Table 3. Chromatographic behaviour of chondroitin sulfuric acid A on ECTEOLA.

Fraction (Molarity Cl^-)	Amount of polysaccharide in % of total quantity eluted in different fractions			
	System A	System B	System C	System D
0.1	3	3	0	0
0.4	—	—	—	0
0.7	5	5	52	40
0.9	—	—	—	54
1.1	16	89	48	5
1.4	70	3	0	1
1.7	6	0	0	0
2.5	—	0	0	0
3.0	0	—	—	—

For experimental data see Table 2.

Table 4. Chromatographic behaviour of heparin (HEP 100) on ECTEOLA.

Fraction (Molarity Cl^-)	Amount of polysaccharide in % of total quantity eluted in different fractions			
	System A	System B	System C	System D
0.1	0	0	0	0
0.4	—	—	—	0
0.7	0	0	0	9
0.9	—	—	—	40
1.1	0	0	66	49
1.4	0	13	34	2
1.7	4	41	0	—
2.5	24	46	0	0
3.0	56	—	—	—
0.5 M NaOH	16	0	0	0

For experimental data see Table 2.

The different behaviour of hyaluronic acid in systems A and B on the one hand and systems C and D on the other hand was probably due to the fact that the pK of the carboxyl groups of hyaluronic acid is 3.2 [21]. The polysaccharide therefore contains hardly any negative charge at a pH below 1.5 (systems A and B). At pH values of 3 and 5 (systems C and D, respectively) the carboxyl group can be expected to be at least partially dissociated and to cause the binding of the polysaccharide to the basic groups of the ECTEOLA column.

The two sulfated polysaccharides studied showed a different behaviour. Chondroitin sulfuric acid A (Table 3) was retained by the exchanger in all four systems. For complete elution of CHS-A the highest chloride concentration was required with system A (HCl), the lowest chloride concentration with system D.

Heparin showed a behaviour similar to chondroitin sulfuric acid A with the four chromatographic systems. In system A heparin was only partially eluted even at 3.0 M HCl. Complete elution of heparin was only obtained by raising the pH (Table 4).

The behaviour of the two sulfated polysaccharides is at first sight rather unexpected. Because of the dissociation of carboxyl groups the polysaccharides would be expected to contain a higher negative net charge at pH values of 3 and 5 (systems C and D, respectively) than at pH-values below 1.5 (systems A and B). Thus one would expect that the polysaccharides would be eluted with a lower chloride concentration at the more acid pH. The reverse was found to be the case.

The sharpest separations of hyaluronic acid, chondroitin sulfuric acid and heparin from each other could be obtained with systems A and B. Since heparin was not completely eluted from the resin with 3 M HCl in system A, it was considered that system B was more suitable for elution of the polysaccharides. System B was therefore studied further.

Fig 3 shows a separation of an artificial mixture of HYA, CHS-A and Hep 120 by step-wise elution. It can be seen that a clear separation of the three polysaccharides into three main peaks was obtained. As demonstrated in

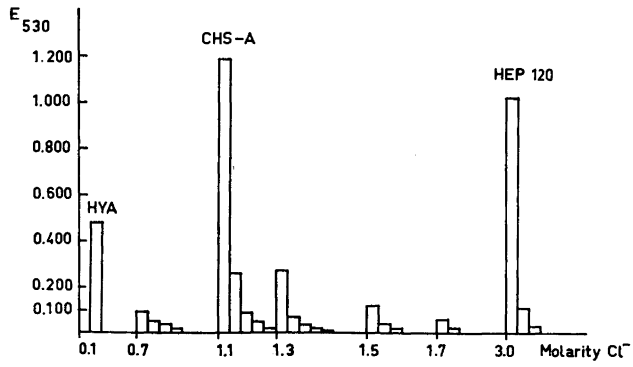

Fig. 3. Fractionation on an ECTEOLA column (1 × 12 cm) of a polysaccharide mixture containing 6 mg HYA, 14 mg CHS—A and 13 mg HEP 120. Fractions 12—15 ml/30 min.

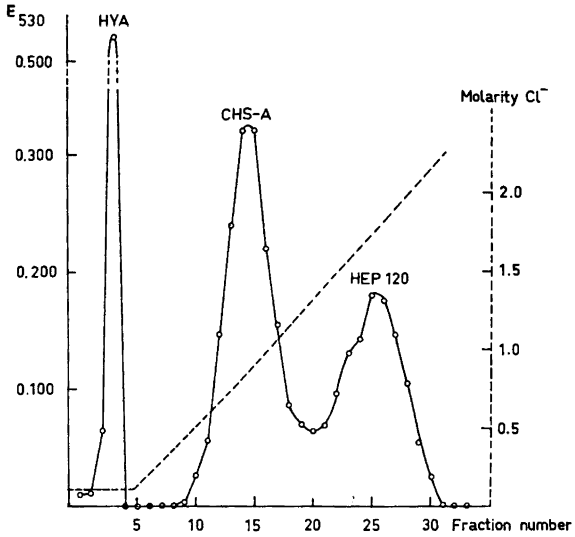

Fig. 4. Gradient chromatogram of the same mixture as in Fig. 3. Reservoir: 400 ml of 3.0 M NaCl—HCl (1:1), mixing vessel: 400 mg of 0.1 M NaCl—HCl (1:1). Fractions ca 20 ml/h. Dotted line = salt concentration.

separate experiments, materials eluted at 0.7, 1.1 and 1.3 M Cl^- originated from the CHS-A preparation, while the HEP 120 contained the material eluted in the 1.5, 1.7 and 3.0 M Cl^- fractions.

A gradient chromatography of a similar mixture of the three polysaccharides is shown in Fig. 4. Again three main peaks, correspondig to HYA, CHS-A and HEP 120 were obtained. The latter two polysaccharides were not separated completely. This behaviour was expected from the behaviour of CHS-A and HEP 120, on individual columns, and from the results obtained in the experiment shown in Fig. 3. The gradient chromatograms clearly demonstrate that the separations obtained by step-wise elution were not due to chromato-

Table 5. Fractionation of heparin into subfractions by chromatography on ECTEOLA.

	amount of material	anticoagulant activity
	mg	units/mg
Starting material	225	110
1.4 M fraction	23	50
1.6 M »	68	85
1.8 M »	30	130
2.0 M »	35	130
2.5 M »	20	140

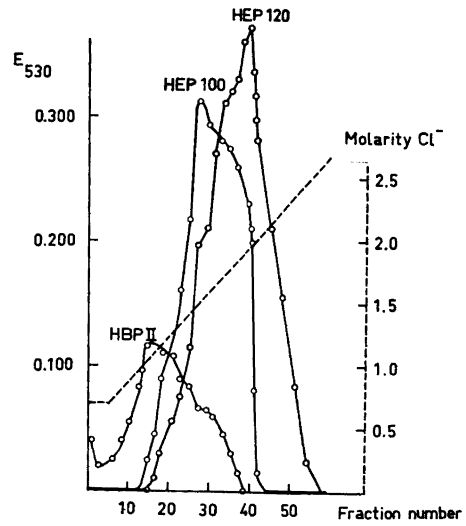

Fig. 5. Chromatography on three separate columns of "heparin" preparations of different anticoagulant activity. The three columns (1 × 5 cm) were eluted simultaneously from the same mixing vessel. Reservoir: 400 ml of 3.0 M NaCl—HCl (1:1), mixing vessel: 400 ml of 0.7 M NaCl—HCl (1:1). Fractions 3 ml/20 min, 12 mg of polysaccharide on each column.

graphic artefacts. However, step-wise elution allowed more clearcut separations and was therefore used in most experiments.

In the experiments described so far, no special attempts were made to obtain subfractions of heparin. However, it was thought of interest to see whether a commercial preparation of this polysaccharide could be further fractionated by chromatography. Such an experiment is described in Table 5.

The fractions obtained differed from each other as regards anticoagulant activity. The most active fractions were those eluted at the highest ionic strength. Fractionation of heparin into materials of different anticoagulant activity has been obtained by several authors [22], the earliest studies in this field being those of Jorpes [23].

In another set of experiments a comparison was made of the chromatographic behaviour of three "heparins" of different anticoagulant activity during gradient elution. HBP II, HEP 100 and HEP 120 were chromatographed on three separate columns of the same dimensions. The three columns were connected with the same gradient system. The results (Fig. 5) indicate that, even though considerable overlapping did occur, the three polysaccharides nevertheless showed distinct differences in their chromatographic behaviour. More material was eluted at higher salt concentrations from the HEP 120 preparation than from HEP 100. A similar difference existed between HEP 100 and HBP II. Thus in general the materials with the highest anticoagulant activities tended to show the highest affinity to the column.

As reported earlier [7] chromatography on ECTEOLA in system B was not found to cause demonstrable inactivation of heparin or loss of sulfate groups from this polysaccharide. It was also found that the recovery of polysaccharides (hyaluronic acid, chondroitin sulfuric acid A and B, and heparin) was close to 100 %. On the other hand losses of some polysaccharides occurred when salts were removed by dialysis.

Chromatography on ECTEOLA with system B has been used for the preparation from mast cell tumors of polysaccharide fractions of different chemical and biological properties [24]. The method was also easily adapted to a microscale which has permitted the fractionation of the polysaccharide from normal peritoneal mast cells in quantities less than 500 μg [25]. When used with the orcinol reaction for the demonstration of polysaccharides in the effluent the separation of 20 μg of HYA, 20 μg of CHS-A and 20 μg of HEP 120 could be followed without difficulty.

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