A CHROMATOGRAPHIC METHOD FOR THE SEPARATION OF ACID MUCOPOLYSACCHARIDES*

by

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Chemical and biological characterization of the acid mucopolysaccharides has been hampered by inadequate methods for separation and resolution of polydisperse preparations. Recently, methods involving electrophoresis[1,2] and paper chromatography[3] have been described for the separation of different acid mucopolysaccharides, but no claim has been made of separation of individual compounds into fractions of different molecular weights. An observation[4] that heparin could be fractionated with a "carrier" amine by counter-current techniques suggested that other acid mucopolysaccharides might similarly be separated, purified or fractionated. The following studies describe a column chromatographic method using aliphatic amines for the fractionation and separation of hyaluronic acid (HA) and chondroitinsulfuric acid (CSA).

METHODS

The mucopolysaccharides used in these experiments were HA from filtrates of a strain of Group A streptococcus, and alkali-extracted cartilage CSA, prepared by methods previously described[5,6]. Both substances were converted into the free acid by treatment with Amberlite IR-120§ in the H^+ form. This is of great importance since the method described is not applicable to the salts of the polysaccharides.

A variety of aliphatic amines as obtained from Armour Laboratories§§ were utilized in these studies. Several supporting agents, including different grades of celite and powdered silica, were tried, but celite (Johns-Manville #545) was found to be most satisfactory. The celite was treated with hot concentrated HCl, thoroughly washed with distilled water and dried at 100°. It was then siliconized by treatment with vapors of a silane preparation (General Electric dri-film #9977, diluted with three parts of benzene) and heated at 160° for 2 hours.

Preparation and elution of column. The siliconized celite, mixed with one half its weight of a solution containing 3% lauryl amine in butyl alcohol saturated with water, was made into a slurry with distilled water which was first slightly undersaturated (approximately 10%) with butyl alcohol. Thereafter, all liquids passed through the column were similarly slightly undersaturated with butyl alcohol. After washing the column with distilled water, the polysaccharide (in the acid form) dissolved in distilled water (saturated with butyl alcohol) was placed on the column and the column washed with water until the eluate was clear. (The initial washing was necessary because

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§§ Obtained through the courtesy of Dr. PAUL DU BROW.

of a loss of some of the amine and 0–35% of the polysaccharide from the column. Although this material could be recovered, conditions for preventing this loss were not found.) The polysaccharide was eluted with solutions of NaCl of increasing concentrations, and finally with 1% NaOH. Columns containing 50 g of celite measured 12 cm in length and 3.75 cm in diameter, and retained 200 to 250 mg of CSA or 100–150 mg of HA. Fractionation was obtained by elution with 500 ml of each concentration of NaCl (from 0.02 to 2.0 M) at a flow rate of about 2–3 ml/minute.

Analyses. Aliquots of the eluents were made strongly alkaline with 5% NaOH and after 3–4 extractions with ether to remove butyl alcohol and lauryl amine, were analyzed for uronic acid[7]. These determinations were related to standard curves constructed on the basis of pure preparations of CSA and HA. Since different optical densities were obtained for equal amounts of the two polysaccharides, the results were only approximations for fractions containing mixtures.

For other analyses it was necessary to remove this amine, NaCl, and concentrate the fractions to small volumes. This was accomplished by dialysis, treatment with Amberlite IR-120 H^+ and IR-400 OH^-, and concentration to dryness *in vacuo* at temperatures not exceeding 50°. The isolated polysaccharide fractions were then reconstituted in water, 0.15 M NaCl or buffer, and the following analyses were carried out: nitrogen by micro-kjeldahl digestion and Nesslerization, sulfate after acid hydrolysis by a nephlometric procedure[8], and viscosities in an Ostwald Viscometer at 30°, 0.15 M NaCl, 0.1 M PO_4 buffer pH 6.8. The viscosity determinations were determined at polysaccharide concentrations varying from 0.1 to 0.5% and 0.2 to 0.9% for HA and CSA respectively. Under the conditions utilized, reduced viscosity does not vary sufficiently with concentration to account for the experimental differences observed. One preparation of radioactive HA containing ^{14}C was utilized and the radioactivity was measured with a windowless, gas-flow chamber. With the methods of analyses for sulfate and radioactivity employed, mutual contamination of 5% of the two polysaccharides could be determined.

EXPERIMENTAL

In an attempt to develop a method employing partition chromatography, a series of preliminary experiments were performed to determine the distribution of the polysaccharides between a water phase and an amine-organic solution under a variety of conditions. It was found in these studies that, although the amine-organic solvent did extract the polysaccharide in the free acid form from a water solution, the presence of a number of substances—celite, powdered silica, glass, sand, and filter paper—which might conveniently be used as supporting media, resulted in a marked change in the phase distribution of polysaccharide. In view of the fact that the studies of partition between two solvent phases are of some interest, they are briefly reported. CSA was used in most experiments since it was readily available. The CSA was demonstrated to be about twice as soluble in the amine-organic phase as was HA. Although experiments with many of these amines resulted in emulsions at the interphase, which caused an inaccuracy of estimations, the results (Table I) indicated marked variability of the partition coefficient, depending on the amine used. Extraction by butyl alcohol containing as little as 0.03% lauryl amine occurred with an apparent maximum extraction at a 1% concentration. Changes in organic solvent systems resulted in variations in amounts of polysaccharide extracted from water. As an example, increasing concentrations of $CHCl_3$ in butyl alcohol progressively decreased the amount of extraction. Other conditions which were noted to affect the distribution were the presence of salts (NaCl above 0.0001 M) buffers, glycine or large amounts of protein in crude preparations of polysaccharide.

In the course of attempts to overcome the apparent adsorption of the mucopolysaccharide-amine complex on the various solid media, it was found that the presence of inorganic salts prevented such adsorption. It was, therefore, decided to attempt to utilize salt solutions as a means of elution of polysaccharide. Such a method was found to be feasible with siliconized celite containing 3% lauryl amine in butyl alcohol as a stationary phase and varying concentrations of NaCl for elutions.

TABLE I

VARIATION OF THE AMOUNT OF CHONDROITINSULFURIC ACID EXTRACTED FROM A WATER SOLUTION BY AMINE-BUTANOL SOLUTIONS

Amine[*]	*Organic phase*[**] *mg per ml*	*Water phase mg per ml*	*Ratio Organic/*H_2O
CSA – 2.0 mg/ml			
Lauryl amine	1.97	0.05	39.0
CSA – 0.67 mg/ml			
Tri-N-butyl amine (5%)	0.10	0.57	0.18
Di-N-butyl amine (5%)	0.0	0.69	—
Tri-ethyl amine (5%)	0.0	0.67	—
CSA – 4.84 mg/ml			
N-octylamine	1.4	3.4	0.41
C 12 Tetramine[***]	3.8	1.0	3.80
T-Triamine[***]	0.3	4.5	0.06
Ethomeen[***] 2 C/13.4	4.1	0.7	5.80
Ethomeen[***] 2 C/22	2.9	1.9	1.50
Ethomeen[***] C/60	0.0	4.7	0.02
Ethomeen[***] 2 C/44	3.0	1.7	1.83

[*] The amine concentration was 3% except where stated.

[**] Determinations of CSA in organic phase was obtained by re-extracting with 5% NaOH solution, washing with ether and performing carbazole color reaction[7].

[***] Designation used by Armour Laboratories.

The results of chromatography are shown in Fig. 1–3 and in Tables II and III. The fractionation of HA (an example is shown in Fig. 1) began at 0.02 *M* NaCl and was complete at 0.15 *M* NaCl. Approximately 81% (70–87%) of HA was eluted by 0.06 *M* NaCl, recovery depending largely upon the loss from the column by washing with water before NaCl elution. The analyses of the HA samples showed no significant differences in composition, but considerable change, almost three-fold, in reduced viscosity (Table II). Increasing reduced viscosities were obtained with progressive elution, suggesting that materials of different molecular weights were being separated.

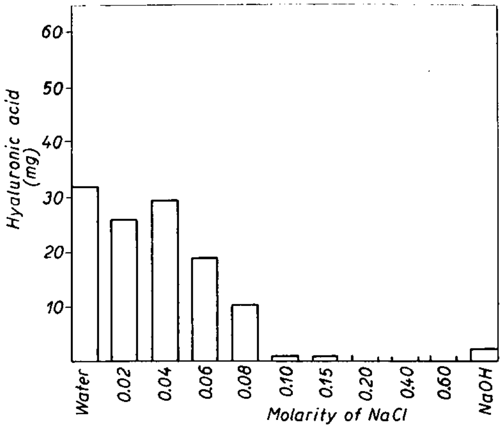

Fig. 1. An example of fractionating hyaluronic acid by column chromatography. The amount of polysaccharide eluted was determined by measurement of uronic acid by the carbazole method. Each change in concentration of NaCl represents approximately 500 ml.

An example of the results of chromatography of CSA is presented in Fig. 2. The CSA was eluted beginning at 0.06 *M* NaCl and, depending upon the amount lost initially by washing, approximately 71% (51–98%) could be recovered after 0.2 *M* NaCl. Chemical analyses of these fractions showed no significant differences in composition. Although the changes were not as marked as those found with HA, increases in viscosity of the CSA fractions were also demonstrated (Table II).

TABLE II

VISCOSITY OF HYALURONIC ACID AND CHONDROITINSULFURIC ACID FRACTIONS OBTAINED BY COLUMN CHROMATOGRAPHY

Hyaluronic acid			*Chondroitinsulfuric acid*		
Fraction	*Viscosity*		*Fraction*	*Viscosity*	
	Exp. 1	*Exp. 2*		*Exp. 1*	*Exp. 2*
[*NaCl*]	$(\eta r-1)/C$	$(\eta r-1)/C$	[*NaCl*]	$(\eta r-1)/C$	$(\eta r-1)/C$
Water	2.64	0.61	Water	0.37	0.30
0.02 *M*	0.89	0.61	0.2 *M*	0.34	0.28
0.04 *M*	1.41	1.13	0.4 *M*	0.40	0.37
0.06 *M*	3.11	1.42	0.6 *M*	0.37	0.46
			0.8 *M*	0.42	
			1.0 *M*	0.56	0.47
Original HA	2.41	0.88	Original CSA	0.41	0.31

Determinations of viscosity were made at 30° in an Ostwald viscometer, 0.15 *M* NaCl, 0.8 *M* PO_4 buffer, pH 6.8.

An experiment using a mixture of HA and CSA is shown in Fig. 3, with the analyses in Table III. A study of these data, particularly of sulfate, radioactivity and viscosity, indicates a separation of the two substances. No sulfate was detectable in the early HA fractions, but was present in the eluate of the initial washing with water, in an intermediate zone and in the CSA fractions. Chromatography of radioactive HA mixed with CSA resulted in maximum specific activity similar to the control HA sample, in the expected peak HA fractions, decreasing activity in the intermediate fractions and none in the region of the CSA fractions. A further indication of separation was revealed by changes in reduced viscosity.

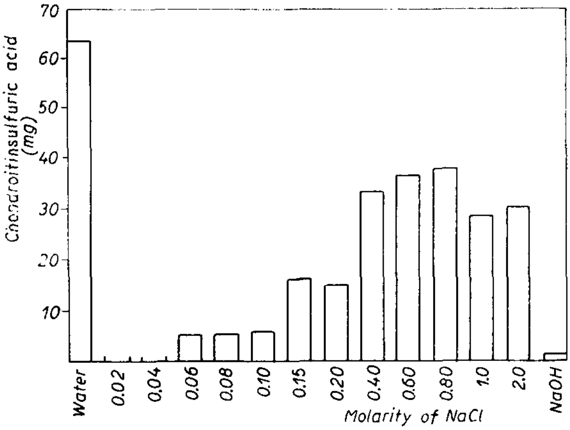

Fig. 2. An example of fractionating chondroitinsulfuric acid by column chromatography. The conditions were similar to those of experiment depicted by Fig. 1.

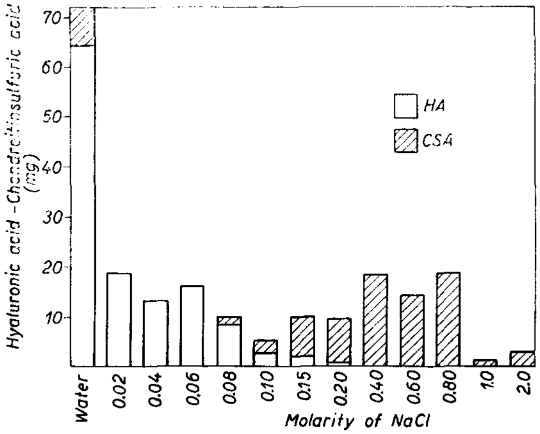

Fig. 3. The separation of a mixture of hyaluronic acid and chondroitinsulfuric acid by column chromatography with conditions similar to experiments shown by previous figures. Analyses of the polysaccharides isolated in these fractions are shown in Table III. The relative proportions of HA and CSA in the fractions containing mixtures were estimated from the SO_4 and radioactivity determinations.

TABLE III

RESULTS OBTAINED FROM A COLUMN CHROMATOGRAPHIC SEPARATION OF A MIXTURE OF HYALURONIC ACID AND CHONDROITINSULFURIC ACID

Fraction *(M NaCl)*	*Nitrogen/polysaccharide**	*Sulfate/polysaccharide*	*Radioactivity* *Specific activity*	*Viscosity* $(\eta_{r}-1)$ *C*
Water	3.5	0.6	43	0.60
0.02	4.0	—	46	0.60
0.04	4.2	—	57	0.77
0.06	3.9	—	52	1.17
0.08	4.2	—	41	1.15
0.10	3.8	1.3	26	0.61
0.15	3.4	3.7	13	0.41
0.20	3.9	4.3	—	0.31
0.40	3.7	4.8	—	0.34
0.60	3.3	4.9	—	0.38
0.80	3.5	4.8	—	0.41
Control HA	(4.0)	(—)	(46)	(0.88)
Control CSA	(2.6)	(4.8)	(—)	(0.39)

* Represents the ratio of nitrogen to polysaccharide as determined by the carbazole method.

DISCUSSION

The experiments involving extraction of CSA and HA by an amine-organic solvent solution and those on heparin[4] demonstrated a distribution of highly polar molecules between water and an organic solvent medium.

The chromatographic studies indicated separation of the CSA and HA principally through measurements of sulfate and radioactivity, but further evidence was found in differences in reduced viscosity of HA and CSA fractions. The chemical determinations were sufficient to demonstrate a separation, and although these agreed with results for control samples of the purified polysaccharides, they did not always agree with theoretical values. This may be explained in part by the relation of values to an arbitrary standard (carbazole determination).

The procedure described seems to be of additional value to previous techniques used for the separation of the acid mucopolysaccharides HA and CSA in that there is a fractionation of these substances into components with different reduced viscosities, probably with different molecular weights. Such resolution of HA was quite evident. Since no apparent alteration of chemical composition occurred and materials of higher viscosities than the original substances were isolated, fractionation and recovery procedures did not seem to degrade the polysaccharides.

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SUMMARY

The results of extraction of chondroitin sulfuric acid in a water solution by various amines in butanol and the effects of certain conditions on the extraction are briefly presented.

A method of column chromatography was developed for fractionating hyaluronic acid and chondroitin sulfuric acid and for separating mixtures of these substances. The materials isolated from the columns showed no differences in composition from purified preparations, but showed differences in reduced viscosity.

RÉSUMÉ

Les résultats de l'extraction de l'acide chondroïtine sulfurique en solution aqueuse par diverses amines en solution butylique et l'influence de certaines conditions sur l'extraction sont exposés brièvement.

Une méthode de chromatographie sur colonne a été mise au point pour le fractionnement des acides hyaluronique et chondroïtine sulfurique et pour la séparation de mélanges de ces substances. Les produits isolés sur colonne ne présentent pas de différences de compositions par rapport à des préparations purifiées, mais présentent des différences dans la viscosité diminuée.

ZUSAMMENFASSUNG

Die Extraktion von Chondroitinschwefelsäure aus seiner wässerigen Lösung durch verschiedene Amine in Butanollösung, sowie der Einfluss gewisser Faktoren auf diese Extraktion werden kurz erläutert.

Der Gebrauch einer neuen chromatographischen Kolonne, welche die Fraktionierung von Hyaluronsäure und Chondroitinschwefelsäure, sowie die Trennung von Mischungen dieser Substanzen ermöglicht, wurde eingeführt. Die aus dieser Kolonne zurückgewonnenen Substanzen zeigten sich, ihrer Zusammensetzung nach, von gereinigten Präparaten keineswegs verschieden, doch traten an ihnen, ihrer niedrigeren Viskosität nach zu schliessen, gewisse Änderungen auf.

REFERENCES

[1] S. GARDELL, A. H. GORDON AND S. AQVIST, *Acta Chem. Scand.*, 4 (1950) 907.
[2] K. G. RIENITS, *Biochem. J.*, 53 (1953) 79.
[3] G. P. KERBY, *Proc. Soc. Exptl. Biol. Med.*, 83 (1953) 263.
[4] A. E. O'KEEFFE, F. M. RUSSO-OLESI, M. A. DOLLIVER AND E. T. STATLER, *J. Am. Chem. Soc.*, 71 (1949) 1517.
[5] S. ROSEMAN, F. E. MOSES, J. LUDOWIEG AND A. DORFMAN, *J. Biol. Chem.*, 203 (1953) 213.
[6] M. B. MATHEWS, S. ROSEMAN AND A. DORFMAN, *J. Biol. Chem.*, 188 (1951) 327.
[7] Z. DISCHE, *J. Biol. Chem.*, 167 (1947) 189.
[8] G. TOENNIES AND B. BAKAY, *Anal. Chem.*, 25 (1953) 160.

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