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A NEW CHROMATOGRAPHIC METHOD FOR THE FRACTIONATION OF HYALURONIC ACID

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Sodium hyaluronate from rooster comb, umbilical cord, bovine vitreous and a special commercial hyaluronic acid preparation, "Healon®" have been fractionated at 4°C on DEAE-Sephacel columns using salt gradients from 0→0.4M NaCl. All samples examined displayed a high degree of polydispersity. The number of well separated fractions observed in these chromatograms ranges from 10-30 depending on the source of the material. A molecular weight distribution effect does not seem to be the main factor controlling this fractionation as no differences could be observed when the limiting viscosity numbers (η) for numerous fractions isolated from the same sample were compared with each other. As no galactosamine, sulfate or phosphate is found directly linked to these fractions, it is suggested that disaggregation of an hyaluronate molecular aggregate could be the basis for this fractionation.

Commercial preparations of hyaluronic acid are increasingly being clinically used as a vitreous substitute in eye surgery with varying degrees of success. In view of the sensitive nature of the human eye to inflammation resulting presumably from the presence of foreign substances, it has become mandatory to devise new methods which are capable not only of assuring uniformity between various hyaluronate preparation; but also of generating a better understanding of the possible structure-function relationship of this polymer.

This mucopolysaccharide was first isolated from bovine vitreous by Meyer and Palmer in 1934 (1) and in subsequent years from a variety of different tissues (2-5). While all the sulfated mucopolysaccharides have been shown to be chemically heterogeneous, hyaluronic acid remains the only known glycosaminoglycan which appears polydispersed, with no proven chemical heterogeneity. Light scattering and other physicochemical studies (6-10) carried out on hyaluronic acid have failed so far to explain the features which could be responsible for the polydispersity observed with this polymer. This drawback results from a lack of proper methodology available for the fractionation of hyaluronate into discrete chemical entities. Several

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investigators have attempted to fractionate hyaluronic acid using DEAE-Celluose (11) and DEAE-Sephadex (12). However, the inherent physicochemical limitations associated with these fractionation procedures have hindered the possibility of obtaining well separated hyaluronate fractions.

In this report, we describe a new chromatographic procedure capable of fractionating for the first time hyaluronic acid from many sources into as many as 10-30 discrete fractions. The high sensitivity and reproducibility of this method makes it the best available procedure for studying small quantities of hyaluronic acid. As little as 300 micrograms of hyaluronate could be accurately fractionated on these columns with high recovery. This technique should serve as a powerful tool for examining the distribution of hyaluronic acid in normal as well as in pathological connective tissues.

MATERIAL

Rooster comb and human umbilical cord hyaluronate were prepared according to the method described by Balazs and Sweeney, 1968 (13). A special commercial preparation of sodium hyaluronate "Healon^{R}" which is sold as a 1% solution of sodium hyaluronate for eye surgery was supplied by Pharmacia AB, Sweden. Vitreous hyaluronate was obtained from calf vitreous. Calf (1-3 months-old) eyes were collected on ice from the local abattoir, immediately following the death of these animals. After the eyeballs had been cleaned from all surrounding tissues, an equatorial cut was made just below the limbus and both anterior and posterior vitreous were collected. These vitreous samples were cleaned from all adhering tissues, homogenized by aspiration through a syringe and centrifuged in a Spinco preparative ultracentrifuge at 20,000 RPM for 30 minutes. The supernatant was dialyzed against several changes of double distilled water. The retentate was deproteinized by shaking at room temperature with chloroform for a period of 12-24 hrs according to a modification of the method of Sevag (14). Prior to chromatography on DEAE-Sephacel, all samples were dialyzed against several changes of distilled water.

METHODS

Column Chromatography:

DEAE-Sephacel (wet particles size: 40-150um) in the preswollen form was supplied by Pharmacia Fine Chemical, Piscataway, N.J. The resin was poured in a beaker, and suspended several times in distilled water in order to remove fine particles. The material was finally suspended in 0.5M NaCl, poured on to a 1.2 x 120cm. column and allowed to settle by gravity. Prior to the application of samples, the column was washed with double distilled water until the effluent became negative for salt when tested with dilute silver nitrate solution. Dialyzed samples containing 5-15mg of hyaluronic acid in a total volume of 100-200 ml were usually applied to the column at a flow rate of 10ml/hr using an LKB microperpex peristaltic pump. Elution was carried out with a linear salt gradient ranging from 0→0.4M NaCl. The flow rate was adjusted to 8ml/hr using the peristaltic pump and fractions of 1.0ml were collected. For fractionation carried out in the presence of urea, the sample was first dialyzed against 7.0M urea, applied at 4^{o}C to a column equilibrated with the same solvent and finally eluted with a salt gradient made up in 7.0M urea. The salt concentration was monitored during the fractionation, using a conductivity bridge. The eluates were analyzed for uronic acid. Carbazole positive fractions were pooled separately, concentrated by ultrafiltration, dialyzed and used in further experiments.

Analytical Procedures:

Hexuronic acid was estimated by the carbazole reaction (15) and the automated procedure described by Balazs et al. (16). Hexosamines were determined by the method of Elson and Morgan (17) as modified by Balazs et al. (18). Protein content was estimated with Folin's phenol method (19) and by measuring absorbance at 260 and 280 nm as described by Warburg and Christian (20). Total sulfate was estimated according to the turbidimetric method described by Dodgson and Price (21) and by the colorimetric procedure described by Terho and Hartiala (22). Inorganic phosphate was measured as described by Baginsky et al. (23).

Paper Chromatography:

Qualitative identification of glucosamine and galactosamine in various fractions was carried out by hydrolyzing samples in 4N HCL for 16 hours at 100°C, degrading the hexosamines to their corresponding pentoses with ninhydrin, (24) separating the various components by paper chromatography using butanol/ethanol/water (4:1:1) as solvent, and detecting the resulting pentoses by staining with silver dip reagent (25).

Viscometry:

Prior to viscosity measurements, all samples were dialyzed against several changes of 0.15 M NaCl. Viscosities were measured at 25°C in Cannon semi-micro dilution viscometers. Intrinsic viscosities were determined by extrapolation of the reduced viscosities to zero concentration.

RESULTS AND DISCUSSION

In previous studies the polydispersity observed with hyaluronic acid was attributed solely to a difference in molecular size between various hyaluronate fractions (11). Our preliminary results do not support this hypothesis. If we consider the fractionation of rooster comb hyaluronate on DEAE-Sephacel (figure I),

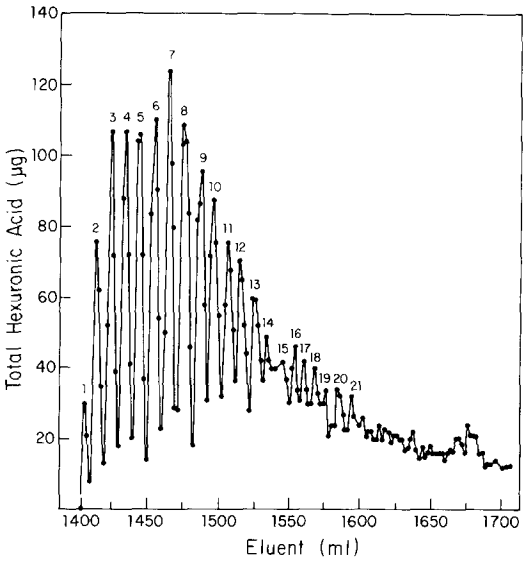

Figure I Fractionation of hyaluronate from rooster comb on DEAE-Sephacel. Column size: 1.2x120cm. Elution is carried out with a linear salt gradient from 0→0.4M Nacl. Flow rate: 8.0ml/hr fractions of 1.0ml collected.

TABLE I

Molecular characteristics of hyaluronate fractions obtained from "Healon^{R}" and rooster comb sodium hyaluronate[a]

Sample	Molar ratio Hexuronic acid / Hexosamines	Molar ratio Sulfate / Hexosamines	Molar ratio Inorganic phosphate / Hexosamines	Intrinsic Viscosity cc/gram (η)
"Healon^{R}"				
Fraction # 1	1.3	0.1	0.3	2550
# 2	1.2	<0.01	<0.01	1800
# 3	1.2	<0.01	<0.01	2320
# 4	1.2	neg	<0.01	
# 5	1.2	neg	<0.01	1920
# 6	1.2	<0.01	0.02	2150
Rooster Comb Sodium hyaluronate				
Fraction # 2	1.0	<0.01	<0.01	2980
# 4	0.9	neg	neg	2100
# 5	1.0			2200
# 7	1.1			2320
# 9	1.1			2380

[a]Each value represents the average of two separate determination performed in triplicate.

a spectrum of well separated hexuronic positive fractions can be observed. No significant differences in limiting viscosity number (η) could be obtained when fractions from both sides of the chromatogram are compared with each other, as shown in Table I. Similar results were obtained when a "Healon^{R}" preparation was chromatographed on DEAE-Sephacel as indicated in figure II. Although the sample appears less polydisperse than regular rooster comb sodium hyaluronate, it nevertheless displayed a considerable polydispersity. Inspection of table I, again revealed no significant differences in limiting viscosity numbers (η) calculated for various hyaluronate fractions regardless of their relative position in the chromatogram. The relative increase in the limiting viscosity number observed with fraction I from both chromatograms is due to the presence of nucleic acid in this fraction as determined by absorbance at 260nm and inorganic phosphate analyses. Based on these observations, it is clear that size(s) may not be the main factor controlling the elution profile obtained for these fractions on DEAE-Sephacel. One possibility is that purified hyaluronate could be an aggregate or network (27) built up of several sub-units held together in equilibrium by proteins, lipids, divalent

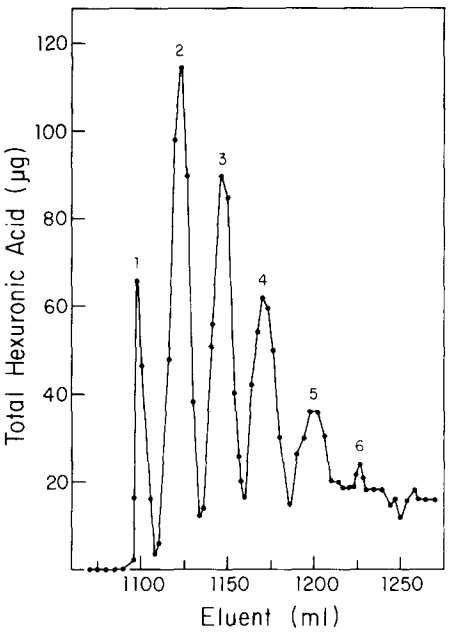

Figure II Fractionation of sodium hyaluronate "Healon^{R} on DEAE-Sephacel. Column size: 1.2x120cm. Elution is carried out with a linear salt gradient from 0→0.4M Nacl. Flow rate: 8ml/hr, fractions of 1.0ml collected.

cations or some ionic forces. The binding of this aggregate on to DEAE-Sephacel could therefore initiate a displacement in equilibrium resulting in the disaggregation of these hyaluronate sub-units at different time and salt concentrations. This hypothesis appear reasonable in light of the observations made by comparing the chromatogram obtained for the chloroform treated bovine vitreous hyaluronate to that of the non-treated one. Deproteinization of bovine vitreous by chloroform is seen to reduce considerably the number of hyaluronate fractions normally obtained from DEAE-Sephacel for non-treated sample. This therefore, indicates that proteins or lipid-like materials may be participating in the organization of hyaluronic acid macromolecules. On the other hand, a totally different concept emerges when isolated hyaluronate fractions are rechromatographed on the same column using these same identical experimental conditions. Figure 3 shows a typical chromatographic profile obtained for the fractionation of a purified umbilical cord hyaluronate on DEAE-Sephacel. When peak #9 from this chromatogram was dialyzed against distilled water and the retentate rechromatographed on the same column, a similar, but not identical pattern was obtained (figure 3B). Indeed, peak #9 upon

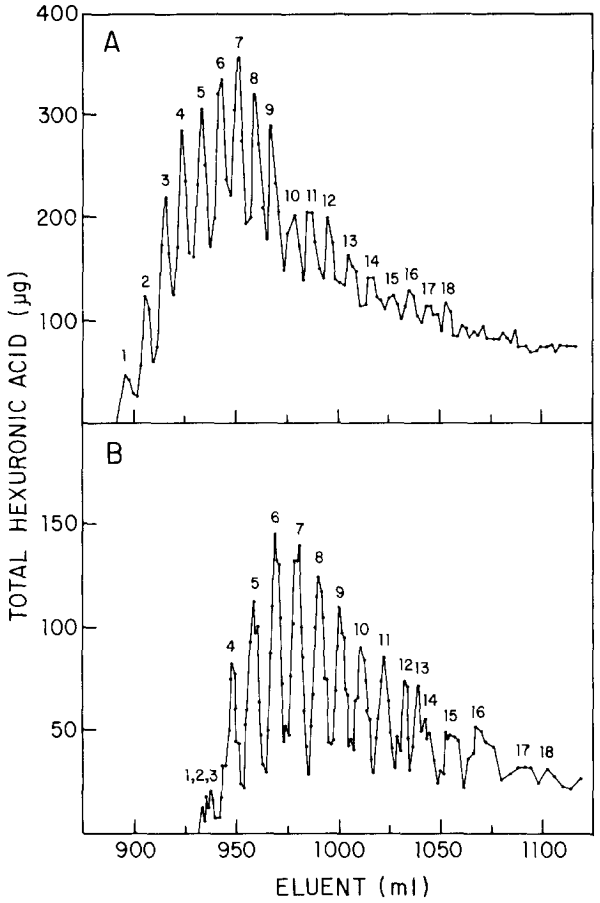

Figure III Fractionation of hyaluronate from human umbilical cord on DEAE-Sephacel column size: 1.2x120cm. Elution is carried out with a linear salt gradient from 0→0.4M Nacl. Flow rate 8ml/hr; fractions of 1.0ml collected. A) Upper panel illustrates the chromatographic profile of the unfractionated sample. B) Lower panel shows the pattern obtained for fraction #9 (from A) rechromatographed on the same column.

rechromatography is found to be inhomogeneous. In fact, there is a great deal of similarity between the chromatographic profile of peak #9 and that obtained for the fractionation of the original sample. Nonetheless, there is a notable difference in these two chromatograms. Numerous fractions which appeared at the beginning of the chromatogram obtained for the original sample are seen to be completely absent from the chromatogram obtained for fraction #9 (Fig. 3B). Their absence suggests that they may have different charge densities from the rest of the fractions in the chromatogram obtained for the unfractionated sample (Fig. 3A). Composition analysis does not seem to provide a basis for the fractionation as no sulfate or phosphate was

found to be associated with these hyaluronate fractions (table I). It is possible however, that varying degrees of lactonization or perhaps some other distinct structural features associated with the uronosyl moieties of these fractions could account for the difference observed in their chromatographic elution profiles.

It is interesting to note that hydrogen bonding does not seem to be participating in the organization of this network or aggregate, since 7.0M urea failed to cause any significant changes in the chromatographic patterns of these samples. By contrast, an increase in temperature to 65°C totally abolished the type of separation usually obtained when these samples are chromatographed at 4°C. This heat effect on hyaluronic acid, which seems to be partly an irreversible process, is being further investigated.

In conclusion, column chromatography on DEAE-Sephacel is found to be the best method for examining the polydispersity associated with hyaluronic acid. Even hyaluronate preparations which were considered to be homogenous, as for example "Healon^{R}" are seen to display considerable polydispersity when examined on these columns. This method is highly reproducible and gives a total recovery of 82-94% based on hexuronic acid. Based on the large number of well separated fractions observed in these chromatograms, it is clear that this technique is superior to all previous procedures used to fractionate hyaluronate . Although, the basis for this fractionation cannot be firmly established at present, it can be utilized to evaluate the polydispersity of hyaluronic acid from a variety of different sources (26).

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