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DETERMINATION OF MOLECULAR-WEIGHT DISTRIBUTION OF AORTA GLYCOSAMINOGLYCANS BY AUTOMATED GEL FILTRATION

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SUMMARY

This report describes an automated gel filtration procedure for estimation of molecular weights of glycosaminoglycans using Technicon sugar analyzer. A Sepharose CL-6B column was used for gel filtration. The method has been applied to determining the distribution of molecular weights of glycosaminoglycans from human aorta. The glycosaminoglycans were fractionated on a Dowex 1 (Cl^-) column prior to gel filtration. On gel filtration, hyaluronic acid resolved into two components with estimated molecular weights of 38,000 and 8000. A molecular weight of 22,000 was estimated for heparan sulfate. Chondroitin 6-sulfate and dermatan sulfate eluted from the column in one peak, suggesting these two glycosaminoglycans have a similar molecular weight, 18,000. The procedure was found suitable for studying the gel filtration behavior of proteoglycans from bovine aorta and lung.

INTRODUCTION

The biologic properties of connective tissue glycosaminoglycans (GAG) are closely related to the physicochemical parameters of these macromolecules. Molecular weights of GAG are, therefore, of particular functional significance. The general methods of determining molecular weights of macromolecules such as diffusion, sedimentation velocity and equilibrium, osmometry and light scattering are complex, time-consuming and require large amounts of material. With the introduction of gel filtration for the separation of macromolecules, Constantopoulos *et al.*[1] and Wasteson[2] used gel chromatography to estimate molecular weights of GAG and obtained results which were in agreement with those obtained by other procedures. These studies describe an automated gel filtration method for the determination of GAG by using a Technicon sugar analyzer, and application of the method in determining the molecular-weight distribution of human aorta GAG. The gel filtration profiles of proteoglycans from bovine aorta and lung were also studied.

MATERIALS

Standard samples of known average molecular weights of hyaluronic acid (molecular weight, 230,000), chondroitin 6-sulfate (molecular weight 40,000), dermatan sulfate (molecular weight, 27,000) and heparin (molecular weight, 11,000), were generous gifts from Dr. J. A. Cifonelli, University of Chicago, Chicago, Ill., U.S.A.

Sepharose CL-6B and 2B were purchased from Pharmacia (Piscataway, N.J., U.S.A.). Reagent grade sulfuric acid was obtained from DuPont (Wilmington, Del., U.S.A.) and orcinol from Nutritional Biochemical Corp. (Division of ICN, Cleveland, Ohio, U.S.A.).

METHODS

Isolation of glycosaminoglycans from human aorta

GAG were isolated from human aortic intima by procedures previously described[3,4]. Briefly, the method consisted of digestion of the tissue with 2% NaOH, followed by papain digestion, precipitation of peptide materials with trichloroacetic acid, filtration through Celite, dialysis of the filtrate to remove trichloroacetic acid, concentration to a small volume and precipitation of GAG with 4 volumes of ethanol in the presence of sodium acetate (1%).

Fractionation of glycosaminoglycans

GAG from human aortic intima were fractionated on a Dowex 1 (Cl^-) column by an automated procedure previously described[5]. Unlike the earlier procedure, the effluent from the column was not dialyzed before it was analyzed because of the unavailability of a beaker dialyzer. Analysis of the effluent without prior dialysis reduces the area of the peak of chondroitin sulfates and dermatan sulfate by 2.3 times due to the presence of NaCl and $MgCl_2$, but does not alter the elution time of the GAG. If quantitation of these GAG is needed, the area of this peak should be multiplied by a factor of 2.3. The fractions corresponding to hyaluronic acid, heparan sulfate, and chondroitin sulfates and dermatan sulfate were collected, exhaustively dialyzed against distilled water and lyophilized.

Isolation of proteoglycans from bovine aorta and lung

Proteoglycans were isolated from bovine aorta, lung pleura and gas exchange tissues as previously described[5–8]. The tissues were extracted with 4.0 *M* guanidinium chloride in the presence of protease inhibitors. The extract was clarified by centrifugation. The density of the clear extract was adjusted to 1.33 g/ml by the addition of solid CsCl and the solution was centrifuged at 100,000 *g* for 40 h at 8°. The bottom third of the gradient was exhaustively dialyzed against 0.5 *M* KCl and proteoglycans were precipitated by the addition of cetylpyridinium chloride (CPC). The proteoglycan–CPC complex was dissolved in 4.0 *M* guanidinium chloride. The solution was recentrifuged at a density of 1.46 g/ml and the major proteoglycan fractions were isolated.

Electrophoresis of glycosaminoglycans

Electrophoresis of GAG was performed on cellulose acetate strips in pyridine–acetate buffer or calcium acetate buffer as previously described[6]. GAG were localized on the strips by staining with alcian blue.

Automated gel filtration procedure

Fig. 1 illustrates the flow diagram of the gel filtration procedure. A chromatographic column (70 × 1.5 cm, 3400 series; Glenco Scientific, Houston, Texas, U.S.A.) filled with Sepharose CL-6B was used in the system. Samples of GAG containing 200–300 μg uronic acid in 0.5 ml of 0.05 *M* NaCl were injected into the column through a three-way valve and eluted with 0.05 *M* NaCl containing 0.02% NaN_3. NaN_3 was used to prevent bacterial degradation of Sepharose and it does not interfere in the orcinol–H_2SO_4 reaction. The rate of elution of the column (0.5 ml/min) was kept constant by negative pressure, *i.e.*, by passing the column outlet tube through the proportioning pump manifold before connecting it to the manifold sample inlet as shown in the flow diagram. The flow-rate of the column outlet pump tubing should be equal to or smaller than the gravity flow-rate of the column, to prevent the column

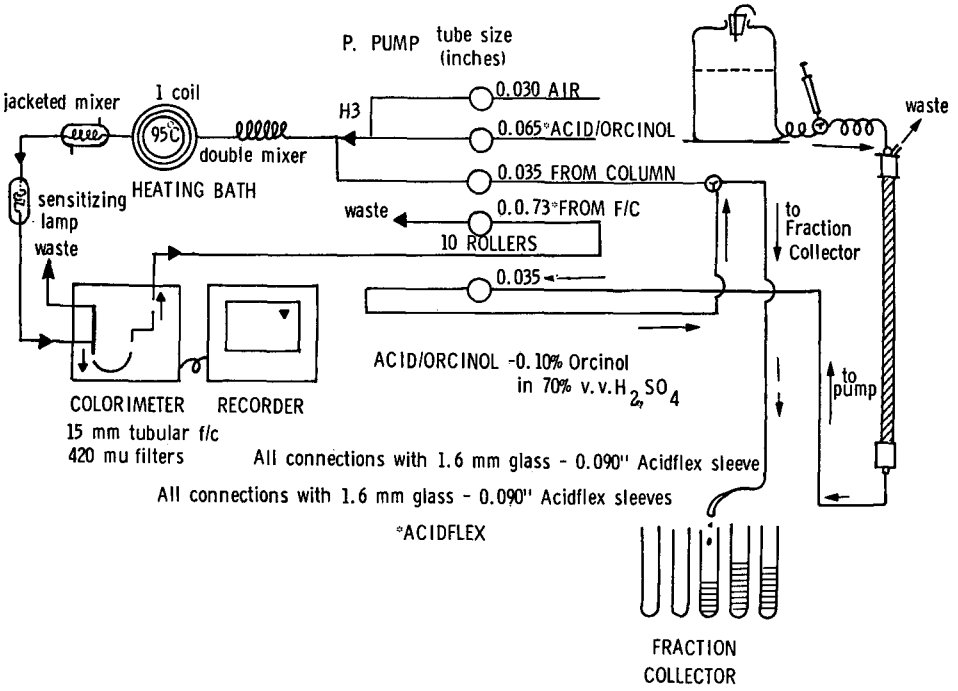

Fig. 1. Flow diagram for the gel filtration of glycosaminoglycans. For glycosaminoglycan chromatography a column (70 × 1.5 cm) filled with Sepharose CL-6B was used. Sepharose CL-2B column (100 × 1.5 cm) was used for proteoglycans. A constant flow-rate of the eluent was maintained by negative pressure by connecting the outlet tube of the column to a proportioning pump tube before connecting it to the sample inlet tube. The sample inlet tube of the proportioning pump was selected to be equal to or smaller than the internal diameter of the column outlet tube which goes through the proportioning pump. For further details refer to text.

from drying. The size of the sample inlet pump tubing of the analyzer should be either equal to or smaller than the column outlet pump tubing which passes through the proportioning pump manifold. If the sample inlet pump tubing is larger than the column outlet pump tubing, air will be drawn into the system and cause an artifactual baseline. The sample waste outlet of the manifold can be connected to a fraction collector if fractions are to be collected for further studies.

The setting of the colorimeter and recorder were: calibration control at 3; sensitivity at damp 2; recorder chart speed 1 in./10 min.

The void volume (V_0) and total volume (V_t) of the columns were determined using *Escherichia coli* and glucose. The K_{av} values were calculated from the equation: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the peak obtained by multiplying the retention time of the peak by the flow-rate of the column. If quantitations of individual GAG are needed they can be calculated from areas under the peaks by triangulation. The chromatographic runs were completed in approximately 4 h.

For gel filtration of proteoglycans a Sepharose CL-2B column (100 × 1.5 cm, 3400 series; Glenco) was used and proteoglycans were eluted from the column by 0.5 *M* sodium acetate pH 5.8 (ref. 9).

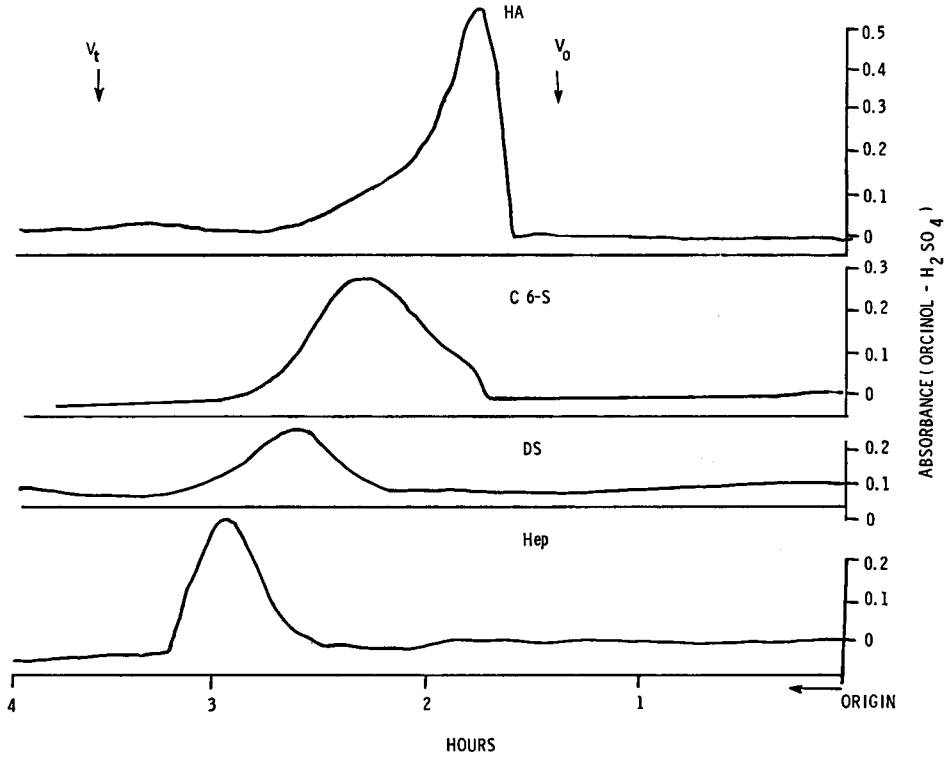

Fig. 2. Gel filtration of standard samples of glycosaminoglycans on a Sepharose CL-6B column. Eluent, 0.05 *M* NaCl. The effluent was analyzed by orcinol–H_2SO_4 in the Technicon sugar analyzer. Abbreviations: HA = Hyaluronic acid; C 6-S = chondroitin 6-sulfate; DS = dermatan sulfate; Hep = heparin; V_0 = void volume of the column determined with *Escherichia coli*; V_t = total volume of the column determined with glucose. K_{av} values of glycosaminoglycans were calculated as described in the text.

RESULTS AND DISCUSSION

Fig. 2 illustrates gel filtration profiles of GAG standards on a Sepharose CL-6B column. Because of better flow-rates, Sepharose CL-6B was used in the column rather than Sepharose 6B. For elution of GAG from the column a low concentration (0.05 *M*) of NaCl was used to reduce the salt effect on gel filtration of GAG[10]. At this concentration of salt the results were very reproducible. Each GAG standard except chondroitin 6-sulfate eluted from the column as a single peak (Fig. 2). Chondroitin 6-sulfate showed a small shoulder at the leading edge of the peak. Hyaluronic acid, although eluting initially as a sharp peak, later showed a delayed elution probably due to its highly viscous property. The K_{av} value of chondroitin 6-sulfate was calculated from the elution time of the major peak. When the K_{av} values of GAG were plotted against their log molecular weights a linear relationship was observed (Fig. 3), and this plot was used to estimate the molecular weights of aorta GAG. Even though the known molecular weights of these GAG are viscosity-average molecular weights and there is evidence for polydispersity in chondroitin 6-sulfate, the K_{av} value of the major peak of this GAG when plotted against its known molecular weight follows the linearity observed for the other standard GAG. The material that eluted in the small shoulder peak of this GAG might not have appreciably contributed to the viscosity-average molecular weight of the GAG.

Fig. 4 illustrates an automated Dowex 1 (Cl^-) column chromatography profile of GAG from human aorta. The GAG clearly resolved into three peaks. Extensive chemical and electrophoretic analyses in previous studies[5,11] indicated that peak 1

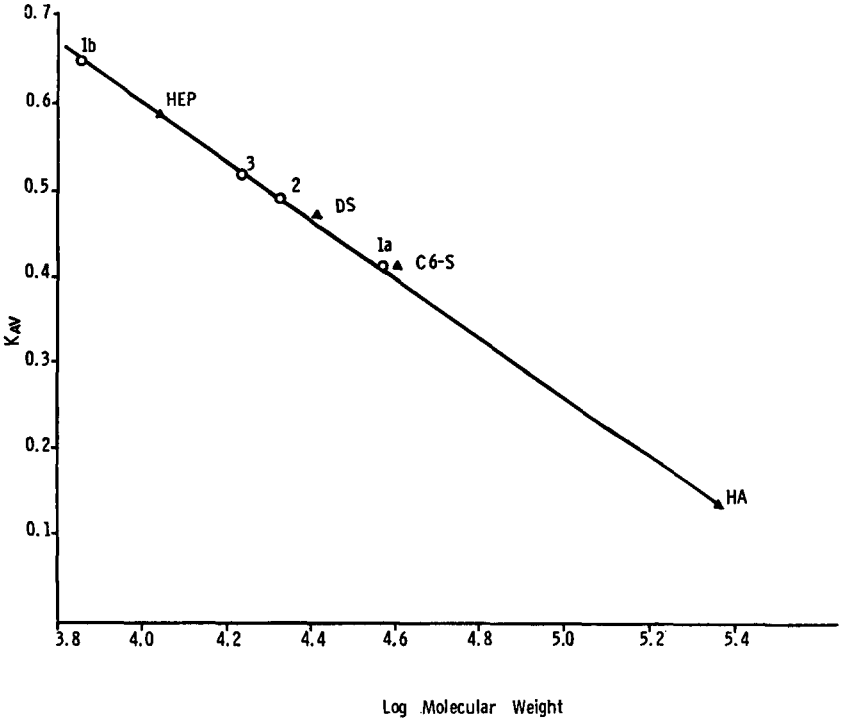

Fig. 3. Relation between K_{av} values and log molecular weights of glycosaminoglycans. Abbreviations as in Fig. 2. Aorta glycosaminoglycans: 1a = hyaluronic acid peak 1 (Fig. 5); 1b = hyaluronic acid peak 2; 2 = heparan sulfate; 3 = chondroitin sulfates and dermatan sulfate.

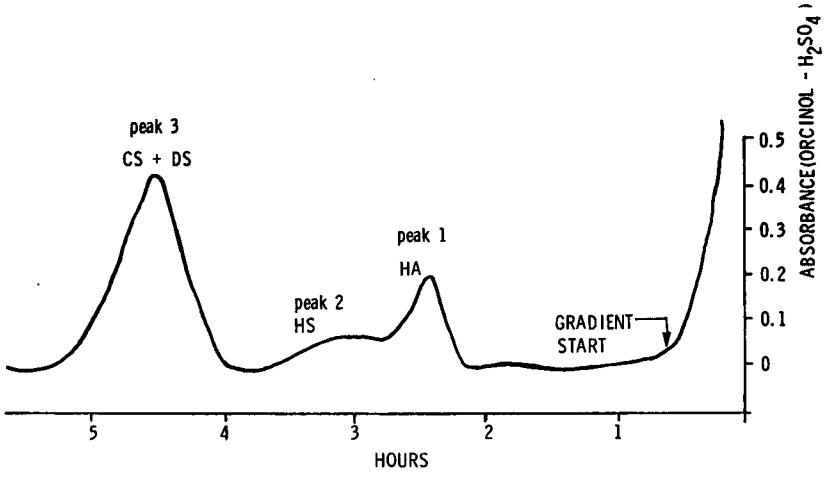

Fig. 4. Chromatography of glycosaminoglycans from human aorta on Dowex 1 (Cl^-) column by an automated procedure described previously[5]. Using a fraction collector, fractions corresponding to peaks were collected, characterized and used for gel filtration (Fig. 5).

represents hyaluronic acid, peak 2 heparan sulfate and peak 3 chondroitin sulfates and dermatan sulfate.

Gel filtration profiles of GAG from aorta on Sepharose CL-6B column are illustrated in Fig. 5. Although hyaluronic acid showed only one spot in cellulose acetate electrophoresis on Sepharose CL-6B column it resolved into at least two peaks with molecular weights 38,000 and 8000. Also there are two minor peaks in the chromatogram and one of them eluted in the void volume of the column. Heparan sulfate gave a broad peak which could be due to its polydispersity and from the elution time a molecular weight of 22,000 was estimated. The chondroitin sulfates and dermatan sulfate which were displaced from the Dowex 1 (Cl^-) column as a single peak (Fig. 4) also eluted from Sepharose CL-6B column as one peak with a K_{av} of 0.52 corresponding to a molecular weight of 18,000. Electrophoretic analysis on cellulose acetate of the fractions of the front, middle and rear of the peak showed two spots in all of the fractions with the major component corresponding to chondroitin 6-sulfate and the minor to dermatan sulfate. It has been previously reported[12] that human aorta dermatan sulfate contains small amounts of chondroitin 4-sulfate and it is possible that the minor component is a hybrid of dermatan sulfate and chondroitin 4-sulfate. Both GAG eluted in a single peak are considered to have the same molecular weight. Of somewhat higher molecular weight, chondroitin 6-sulfate has been isolated from other tissues[4]. The molecular weight of hyaluronic acid from human aorta observed in this study is relatively lower than hyaluronic acid isolated from other tissues[4]. Whether the GAG from human aorta are of lower molecular weight or whether degradation of these GAG might have occurred either before necropsy or during isolation needs further study.

Gel filtration behaviors of proteoglycans from bovine aorta and lung are shown in Fig. 6. Since some of the proteoglycan fractions eluted from Sepharose CL-6B column in the void volume, a Sepharose CL-2B column was used. To minimize intrinsic aggregation[13] a somewhat higher concentration of salt solution was used for elution of proteoglycans from the column.

The chondroitin sulfate–dermatan sulfate proteoglycan isolated from bovine

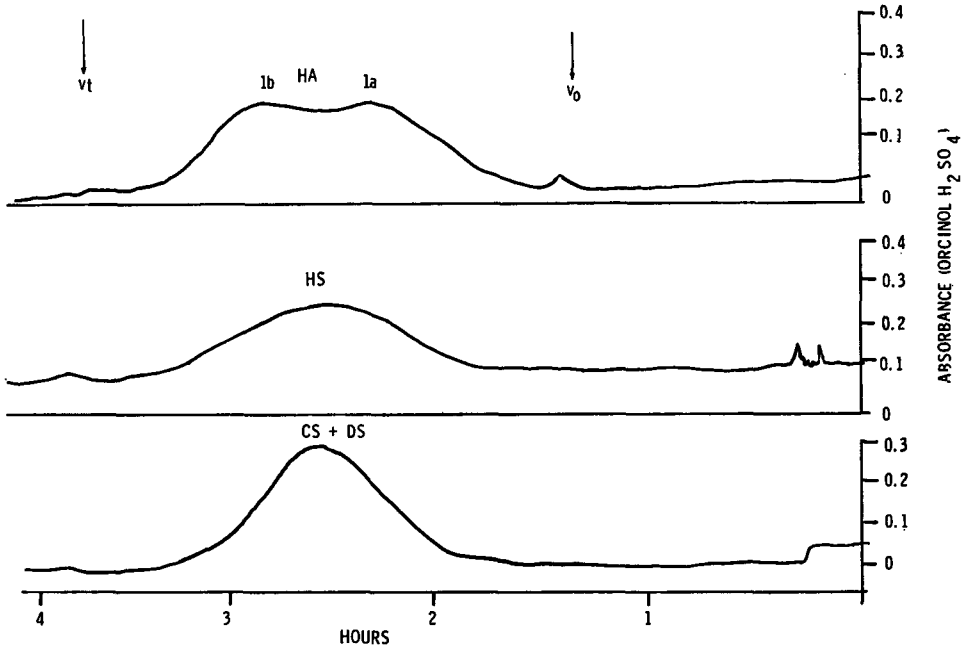

Fig. 5. Gel filtration of glycosaminoglycans from human aorta. The glycosaminoglycans fractions were obtained after fractionation of the mixture as described in Fig. 4. Hyaluronic acid (HA) showed multiple peaks, whereas heparan sulfate (HS), chondroitin sulfates (CS) + dermatan sulfate (DS) gave single peaks. The molecular weights of the glycosaminoglycans were estimated from their K_{av} values (Fig. 3).

aorta by the procedure of Oegema *et al.*[14] appears to be of higher molecular size than that which we previously isolated[6] by the procedure of Sajdera and Hascall[15] without protease inhibitors in the extraction medium. The proteoglycan isolated by the former procedure eluted in the void volume of the column even on Sepharose CL-2B. The proteoglycan from lung pleura eluted from the column as a single peak with K_{av} 0.18. The GAG of this proteoglycan was tentatively identified as heparan sulfate by cellulose acetate electrophoresis after alkali degradation of the proteoglycan. The proteoglycans from lung gas-exchange tissue resolved into at least two peaks on Sepharose CL-2B with the major peak (peak II) having K_{av} 0.34 and the minor peak (peak I) with K_{av} 0.05. The GAG of the major peak was tentatively identified as heparan sulfate and the minor peak as chondroitin sulfate and dermatan sulfate.

The automated gel filtration method described above affords a rapid and reliable method of estimating molecular weights of GAG and proteoglycans in microgram quantities. It has been shown to be useful in determining the distribution of molecular weights of GAG and proteoglycans from arterial and lung tissues. Application of the Technicon sugar analyzer to gel filtration analysis of proteoglycan and GAG is a significant aid to methods currently in use.

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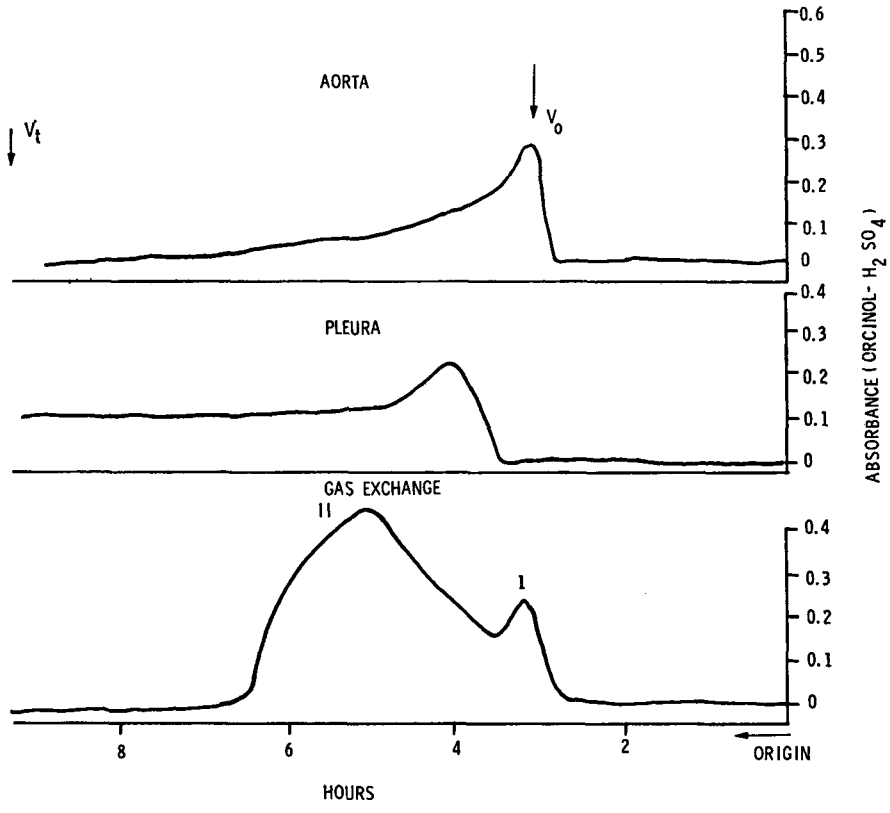

Fig. 6. Gel filtration of proteoglycans from bovine aorta and lung on a Sepharose CL-2B column.

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