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Note

Calibration of agarose columns for gel chromatography with commercially available dextran fractions

Application to the measurement of distributions of molecular radii of glycosaminoglycans

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Changes in the distribution of molecular sizes of the proteoglycans have been proposed as the bases of many biological phenomena involving the connective tissues. Critical testing of these hypotheses has lagged because of the difficulties in the characterization of such distributions. Gel chromatography on calibrated columns is a convenient procedure for this purpose. Wasteson[1] used Sephadex G-200 gel to ascertain optimal conditions for such analyses and calibrated his columns with carefully characterized fractions of chondroitin sulphate[2]. Most laboratories are not prepared for the extensive effort required to prepare such reference materials. End-labelling of glycosaminoglycans by reduction with standardized borotritiide has been proposed as a method of avoiding the problem of column calibration[3]. In our hands, however, molecular weights determined by this method were lower than those obtained from columns calibrated with Wasteson's fractions.

An alternative simple method of calibration was suggested by the commercial availability of dextran fractions with known distributions of molecular weights. A test of this approach is described here with application to the analysis of chondroitin sulphates and keratan sulphates prepared from the proteoglycans of human intervertebral discs.

MATERIALS AND METHODS

Biopolymers

The "rectified native dextran" used for the determination of the void volume of columns was a gift from Dr. Kirsti Granath (Pharmacia, Uppsala, Sweden). Dextran T fractions (Pharmacia, Montreal, Canada) were the gift of Dr. D. E. Brooks of this department. Tritiated water (New England Nuclear, Dorval, Canada) was diluted with 0.02% (w/v) NaN_3 to an activity of 50 μCi/ml. Well characterized fractions of chondroitin sulphate were the gift of Dr. Åke Wasteson (Department of Medical Chemistry, Royal Veterinary College, Uppsala, Sweden): fraction 1, r_s 5.85 nm, fraction 3, r_s 4.35 nm, fraction 8, r_s 3.30 nm, fraction 10, r_s 1.80 nm, where

r_s is the Stokes radius calculated from distribution coefficients on a calibrated Sephadex G-200 column[2].

Glycosaminoglycans

Chondroitin sulphate and keratan sulphate were prepared from proteoglycans isolated from human intervertebral disc using methods described elsewhere[4].

Analytical methods

Dextrans and keratan sulphate were estimated by the phenol–sulphuric acid procedure[5], using anhydrous D-galactose (C grade, Calbiochem, La Jolla, Calif., U.S.A.) as a standard. Chondroitin sulphate was estimated by the carbazole–boro-sulphuric acid reaction[6], using sodium glucuronate monohydrate (Corn Products Refining Co., New York, N.Y., U.S.A.) as a standard. Tritium radioactivity was measured by the addition of 5 ml aqueous counting scintillant (ACS, Amersham/Searle, Arlington Heights, Ill., U.S.A.) to a 500-μl sample in a polyethylene Mini-LSC-vial (New England Nuclear, Lachine, Canada) and counting by a channels-ratio method in a Unilux II liquid scintillation counter.

Gel chromatography

Bio-Gel A-1.5 m agarose (Bio-Rad Labs., Richmond, Calif., U.S.A.) was suspended in 0.20 *M* NaCl containing 0.05 *M* Tris–HCl buffer (pH 7.0) and 0.02% (w/v) NaN_3 and packed to a depth of 900 mm under a head of 300 mm in a 16 × 1000 mm column (Pharmacia, 16/100). Since azide interferes with the formation of the chromogen in the hexuronate reaction, this salt was omitted from the buffer when these analyses were to be performed. All solutions applied to the column were filtered through a 5-μm membrane (Millipore, Bedford, Mass., U.S.A.). The column was eluted by upward flow at room temperature (about 24°); 5-ml fractions were collected using either a volumetric siphon (RadiRac; LKB, Stockholm, Sweden) or drop counter (85 drops, UltroRac 7000, LKB). The tube count began upon application of the sample. The use of a column was discontinued when the flow-rate fell below 10 ml/h.

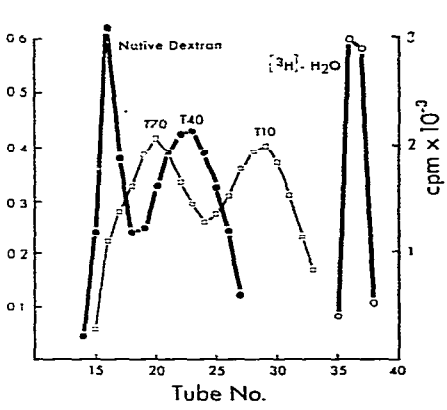
For such relatively monodisperse species as water or native dextran, peaks were located to the nearest 0.1 tube from the plots of the elution profile. With the Dextran T fractions, only the maximum was used.

RESULTS AND DISCUSSION

Columns of Bio-Gel A-1.5 m gave stable beds with flow-rates between 5 and 7 ml cm^{-2} h^{-1} over several months, provided the buffer was degassed and both buffer and sample were freed of small particles by filtration.

A rectified native dextran similar to that used by Wasteson[1] was found to give a sharp elution profile permitting location of the void volume (V_0) with a standard deviation of 0.08 tube number. The total volume (V_t) measured with tritiated water showed a standard deviation of 0.86 tube number. Typical elution profiles of these compounds are shown in Fig. 1.

The partition coefficients (K_{av}) for macromolecules were calculated from their elution volumes (V_e) using the expression of Laurent and Killander[7]:



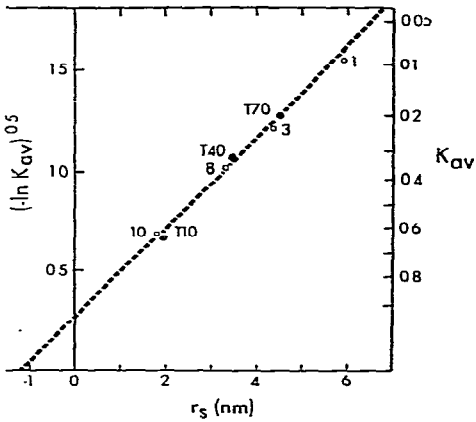


Fig. 1. Calibration of agarose columns. ●, Native dextran (0.5 mg) plus Dextran T40 (1 mg); □, Dextran T10 plus Dextran T70 (1 mg each); ○, or tritiated water (5 μl) dissolved in 0.5 ml 0.2 *M* NaCl containing 0.05 *M* Tris–HCl (pH 7.0) were applied to a 16 × 900 mm column of Bio-Gel A-1.5 m and eluted with the same buffer. Fractions (5 ml) were collected and analyzed for either hexose (dextrans) or radioactivity (tritiated water).

Fig. 2. Partition of various macromolecules on an agarose column. The polymer was applied to a pre-calibrated 16 × 900 mm column of Bio-Gel A-1 5 m, in 0.5–1.0 ml of 0.2 *M* NaCl containing 0.05 *M* Tris–HCl (pH 7.0) and eluted with the same buffer. Fractions (5 ml) were collected and analyzed for either hexose (dextrans) or hexuronate (chondroitin sulphates). The following materials were used: ●, Dextran T fractions (1 mg) T10, T40 and T70; ○, chondroitin sulphate standards (0.4–1.2 mg) 1, 3, 8 and 10 (see ref. 1). The mid-point of the elution profile was used to calculate K_{av}; data were plotted by the method of Siegel and Monty[13]. The line represents the line of best fit to the Dextran T fractions.

$$K_{av} = (V_e - V_0)/(V_t - V_0) \quad (1)$$

Stokes radii rather than molecular weights were used as the basis for calibration because molecular size rather than molecular weight *per se* appears to be the fundamental basis for fractionation on gel columns[8,9].

This work was begun with the assumption that proteins of known Stokes radii would provide convenient monodisperse macromolecular species for the calibration[8]. The proteins showed an unexpected dispersion about the line of best fit and appeared larger than chondroitin sulphates of supposedly equivalent Stokes radii.

The commercial availability of well-characterized fractions of dextran (Dextrans T, Pharmacia) suggested a convenient alternative method of calibration. Each fraction is supplied with a distribution of molecular weights. Even though these preparations are highly polydisperse, each distribution shows a well-defined mode of the molecular weight of which can be readily estimated from the graph supplied. The Stokes radius (nm) corresponding to this molecular weight ($\overline{MW}$) were calculated using the relationship[10]

$$r_s = 0.0332\,(\overline{MW})^{0.463} \quad (2)$$

The equation had been derived from the published data relating molecular weight to diffusion constant for dextrans[11] and the relationship between Stokes radii and diffusion constants for rigid spheres[12]. Although the elution profiles of these

dextrans on Bio-Gel A-1.5 m were broad, each showed a well-defined maximum (see Fig. 1) from which the partition coefficient corresponding to the most abundant species could be calculated. The relationship between such partition coefficients and Stokes radii is shown in Fig. 2. The partition coefficients of the three dextrans (T10, T40, and T70) for repeated runs on several columns showed standard deviations of 0.013, 0.018 and 0.011, respectively. The average values are shown in Fig. 2 as well as the line of best fit to these values.

Precision of the method

The calibration curve of Fig. 2 was used to calculate the apparent Stokes radii of the chondroitin sulphate fractions. These results are compared with the published values in Table I. The observed and published values differed by less than 0.2 nm for each of the four preparations. Thus, the calibration of agarose columns with Dextran T fractions gave values for the Stokes radii of glycosaminoglycans in reasonable agreement with those obtained using columns calibrated with Ficoll standards[2].

Routine procedure

Columns were calibrated routinely by the application of two mixtures in sequence. The first contained rectified dextran, Dextran T40 and tritiated water; the second, Dextrans T10 and T70. The first mixture was re-run at least once every two weeks or every fifth to tenth sample to ascertain the stability of the bed. The partition coefficients of each of the Dextran T fractions were calculated as well as the line of best fit to a Siegel–Monty[13] plot *i.e.* to $(-\ln K_{av})^{0.5}$ *vs.* r_s. The coefficients for the line were used to calculate values of K_{av} corresponding to each r_s value, using eqn. 1. Thus, a plot of tube number against Stokes radius, similar to that shown in Fig. 3, was prepared to calibrate each column. The sharp rise for radii above 7 nm determines the effective upper limit for fractionation on this gel. Reasonable estimates of radii could be made between 1 and 6 nm. In this manner, the average Stokes radius corresponding to each tube was estimated.

Description of the distributions

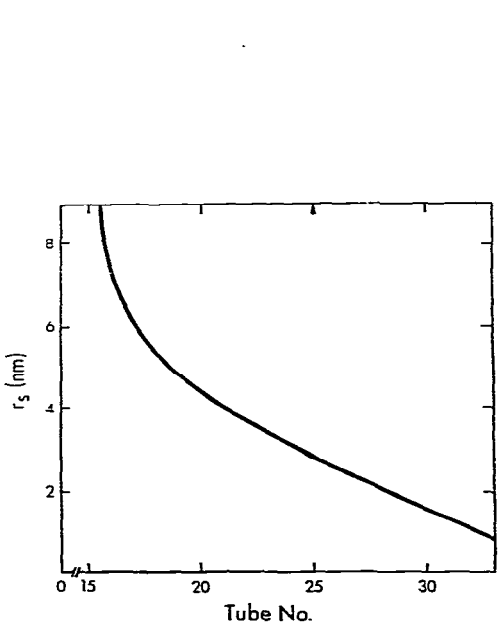
The method was used to describe the distributions of Stokes radii for chondroitin sulphates and keratan sulphates prepared from proteoglycans isolated from human intervertebrial discs by CsCl density-gradient ultracentrifugation[4]. The re-

TABLE I

COMPARISON OF PUBLISHED AND OBSERVED STOKES RADII OF CHONDROITIN SULPHATE FRACTIONS

The published values are those of Wasteson[2]. The observed values were calculated from the elution volume of fractions on an agarose column calibrated with Dextrans T10, T40 and T70.

Chondroitin sulphate fraction No	r_s	
	Published	*Observed*
1	5.85	5.70
3	4.35	4.27
8	3.30	3.40
10	1.80	2.00



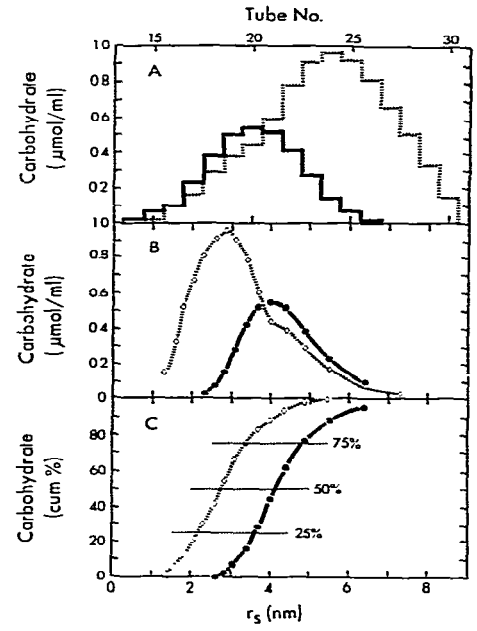


Fig. 3. A typical calibration curve used to determine the Stokes radius corresponding to the tube number of each fraction. The method of calculation is described in the text.

Fig. 4. The distribution of molecular radii of typical glycosaminoglycan preparations. Chondroitin sulphate containing 3.7 μmoles hexuronate (———) or keratan sulphate containing 7.4 μmoles hexose (- - -) from a single proteoglycan preparation were applied separately to a 16 × 900 mm column of Bio-Gel A-1.5 m, previously calibrated with native dextran, Dextrans T10, T40 and T70, and tritiated water. The 5-ml fractions collected were analyzed for hexuronate and hexose, respectively, and gave the distributions shown in A. By the use of a calibration curve, similar to that shown in Fig. 3, the average Stokes radius of each fraction was calculated to give the distributions shown in B. The carbohydrate analyses of the tubes in each peak were summed and the analysis cumulated up to each tube were expressed as a percentage of the total. Plots of the cumulated distributions, shown in C, were used to locate the divisions between the quartiles of the distribution, labelled 25%, 50% and 75% on the graph.

covery ± standard deviation of the hexuronate of the chondroitin sulphates from the columns was 99.5 ± 4.6 (n = 10); that of the hexose of the keratan sulphates was 109 ± 16 (n = 9), the lesser precision of the latter reflecting, in part, the lesser sensitivity and lower precision of the hexose procedure.

Typical elution profiles for the two glycosaminoglycans are shown in Fig. 4A. The distributions of radii calculated from these profiles are shown in Fig. 4B. The distributions were asymmetrical, with a relatively greater abundance of molecules with radii above the mode. The symmetry could be improved by the use of log r_s as abscissa, as discussed elsewhere[4]. Plots of the data for many of the keratan sulphate preparations suggested a bimodal distribution of radii, as may be seen in Fig. 4B, but the separation of the two fractions was never clear-cut. The possibility of two distinct entities in this fraction requires further investigation. To facilitate description of the distributions, the cumulative carbohydrate content, expressed as a percentage of the total, was plotted against the radii. Smooth sigmoid curves resulted (Fig. 4C). Each distribution was divided into quartiles and the radii corresponding to the inter-

TABLE II

REPRODUCIBILITY OF THE DISTRIBUTIONS

Replicate estimates of the distribution of molecular radii of several chondroitin sulphate and keratan sulphate preparations were made as described in Fig. 4.

Glycosaminoglycan	*Preparation No.*	r_s *at interquartile divisions*		
		25%	*50%*	*75%*
Chondroitin sulphate	1	3.50	3.96	4.68
		3.50	4.01	4.88
	2	3.39	3.80	4.34
		3.39	3.78	4.40
	3	3.32	3.77	4.35
		3.40	3.87	4.48
Keratan sulphate	1	1.89	2.31	3.00
		1.89	2.39	3 12
	2	2.40	2.90	3.50
		2.40	3.02	3.75
		2.23	2.82	3.50
	3	2.19	2.75	3.40
		2.25	2.88	3.63

quartile divisions were recorded. Such values from replicate runs are given in Table II. Agreement within the anticipated uncertainty of the method, 0.2 nm (Table I), was found in all but 2 of the 18 measurements.

CONCLUSION

The proposed method of calibration does not represent the desirable comparison with an absolute reference standard. No such method has yet been devised. However, as judged by the results with carefully characterized chondroitin sulphate standards, the values agree well with other indirect methods. The distributions have been described by arbitrary criteria, but in view of the asymmetry and the difficulty in transforming the data to a gaussian distribution the use of such description provides a practical and unbiased solution to the problem of summarizing data.

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