CHROM. 5319

A METHOD FOR THE DETERMINATION OF THE MOLECULAR WEIGHT AND MOLECULAR-WEIGHT DISTRIBUTION OF CHONDROITIN SULPHATE

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(Received February 23rd, 1971)

SUMMARY

A simple micro-method for the determination of molecular weights of chondroitin sulphate has been developed. Columns of Sephadex G-200 and Sepharose 6B were calibrated with fractions of chondroitin sulphate, the molecular weights of which had been determined by independent methods. The use of the calibrated columns for determining the molecular weights and molecular-weight distributions of unknown samples of chondroitin sulphate by gel chromatography is described. The effects of variations in the ionic strength of the eluant were studied. For the average of duplicate determinations of the molecular weight ($\bar{M}_w$) the 95% confidence interval was $\bar{M}_w \pm 3\%$.

INTRODUCTION

With the development of gel chromatography[1], a useful tool for the separation of macromolecules became available. It has been extensively employed in preparative procedures and has also been used for analytical purposes[2]. The application of this technique for the molecular-weight determination of proteins has been reported by several authors[3–5]. Furthermore, the method has been successfully applied to the molecular-weight distribution analysis of polydisperse systems, including hydrophobic[6,7] as well as water-soluble polymers[8,9].

A large number of methods have been employed for the molecular-weight determination of chondroitin sulphate, one of the glycosaminoglycans of connective tissue. These methods include ultracentrifugation[10–13], osmometry[14,15], light scattering[14,16,17] and chemical analysis[15,18]. The various drawbacks of these techniques have emphasized the need for a new, simple, rapid and inexpensive method for small amounts of material.

In the present investigation a method based on the principle of gel chromatography has been developed for the molecular-weight and molecular-weight distribution analysis of chondroitin sulphate. The method has been outlined previously by WASTESON[19] and, independently, by CONSTANTOPOULOS *et al.*[20].

MATERIALS AND METHODS

The preparation and characterization of chondroitin sulphate fractions have been described in a previous publication[21]. Eleven essentially monodisperse fractions were obtained with molecular weights ranging from 2,400 to 36,000. Some relevant physicochemical data are recapitulated in Table I.

TABLE I

PHYSICOCHEMICAL DATA FOR FRACTIONS OF CHONDROITIN SULPHATE[a]

Chondroitin sulphate fraction No.	*K_{av} in 0.2 M NaCl*[b]		*Stokes' radius (r_s) in 0.2 M NaCl (Å)*[c]	*Molecular weight*[d]
	Sephadex G-200	*Sepharose 6 B*		
1	0.11	0.39	58.5	35,800
2	0.20	0.45	49.5	25,400
3	0.28	0.51	43.5	19,200
4	0.34	0.55	39.5	16,500
5	0.41	—	35.0	13,900
6	0.47	—	32.0	11,100
7	0.30	—	42.0	18,300
8	0.45	0.59	33.0	12,400
9	0.61	0.68	25.0	8,600
10	0.74	0.77	18.0	4,800
11	0.84	0.85	12.5	2,400

[a] See also Wasteson[21].
[b] Calculated according to LAURENT AND KILLANDER[20].
[c] Calculated by use of r_s values of Ficoll fractions, as described previously[21].
[d] Calculated as $\frac{1}{2}$ [$\bar{M}_{w(s/D_{app})}$ + $\bar{M}_{w(eq)}$] (ref. 21), except for the value of fraction 7, expressing $\bar{M}_{w(s/D_{app})}$ only.

Three well-characterized fractions of Ficoll, No. XIII, XV and XVI with Stokes' radii 49.0, 34.5 and 26.4 Å, respectively, were available in our laboratory[22].

Chondroitin sulphate was isolated from various sources (see below) by digestion with papain[23] followed by precipitation of the liberated polysaccharide with cetylpyridinium chloride from 0.3 *M* NaCl[24]. ^{35}S-labelled chondroitin sulphate was prepared from slices of rat costal cartilage which had been incubated *in vitro* with [^{35}S]sulphate[25]. The specific activity of the preparation was approximately 1000 c.p.m./μg uronic acid.

Chondroitin-sulphate proteoglycan (PPL) was prepared from bovine nasal septa according to GERBER *et al.*[26]. Dextran gel (Sephadex G-200) and agarose gels (Sepharose 6B and 2B) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Uronic acid was determined by the carbazole method of BITTER AND MUIR[27] or by an automated modification of this method[28]. ^{35}S-activity was measured in a Beckman LS-250 liquid scintillation counter with a dioxane–naphthalene–PPO mixture as the scintillation medium.

Analytical gel chromatography on Sephadex G-200 was performed at 4° on a 0.8 × 63.5 cm column essentially as described previously[21]. Generally, 100–200 μg of chondroitin sulphate or 1 mg of Ficoll were applied to the column, which was

eluted with 0.2 *M* NaCl at a flow rate of about 3 ml·cm^{-2}·h^{-1}. The effluent volume was determined by gravimetry, and the effluent fractions (0.7–0.9 ml) were analysed for uronic acid or ^{35}S-activity.

Analytical gel chromatography on Sepharose 6B was carried out in a similar manner; in this case 200–300 μg of polysaccharide were applied to the column (1.3 × 79 cm).

The void volumes (V_0) and the total volumes (V_t) of the columns[29] were determined by chromatography of a high-molecular-weight fraction of dextran ($\bar{M}_w$ 12 × 10^6) and of tritiated water (Radiochemical Centre, Amersham, Great Britain), respectively[30,31].

The experimental conditions were also extended to include gel chromatography at different ionic strengths and at different concentrations of polysaccharide.

RESULTS

Chromatographic conditions

Effect of ionic strength. Considerable variation in the elution pattern was observed when a fraction of chondroitin sulphate (fraction 3, $\bar{M}_w$ 19,200 (ref. 21)) was chromatographed on the Sephadex column at different ionic strengths (Table II). At low ionic strength the polysaccharide emerged early in the eluate, indicating that the chondroitin sulphate molecules were extended. Also, at low ionic strength ($I < 1 \times 10^{-3}$) the chromatograms tended to become broad and distorted, especially when increased amounts of polysaccharide were applied to the column. With increasing ionic strength the elution of the polysaccharide was progressively retarded, indicating an increased coiling of the molecules. At $I > 0.4$ no further increase in K_{av} was noted. Qualitatively similar results have been obtained with heparin[32]. The variations in the elution pattern of chondroitin sulphate were not due to changes

TABLE II

CHROMATOGRAPHY OF FICOLL AND CHONDROITIN SULPHATE ON SEPHADEX G-200 AT DIFFERENT IONIC STRENGTHS

Ionic strength	K_{av}[a] *for*		r_s *for chondroitin sulphate fraction 3 (Å)*[b]
	Ficoll fraction XV	*Chondroitin sulphate fraction 3*	
5 × 10^{-5}	0.41	[c]	[c]
2 × 10^{-4}	0.42	0.14	55
5 × 10^{-4}	0.42	0.14	55
5 × 10^{-3}	0.41	0.15	53
5 × 10^{-2}	0.42	0.21	48
1 × 10^{-1}	0.42	0.24	46
2 × 10^{-1}	0.42	0.27	44
3 × 10^{-1}	0.41	0.28	43
4 × 10^{-1}	0.39	0.29	43
1 × 10^{0}	0.42	0.29	43

[a] Calculated according to LAURENT AND KILLANDER[29].
[b] Calculated by use of r_s values of Ficoll fractions, as described previously[21].
[c] At ionic strengths < 1 × 10^{-4} the calculations of K_{av} and r_s of the chondroitin sulphate were precluded by the markedly irregular chromatographic behaviour of the latter.

of the properties of the gel, since the K_{av} values of Ficoll were not influenced by the ionic strength (Table II). An estimate of the size of the chondroitin sulphate molecules at the different ionic strengths (Table II) was obtained by comparison with Ficoll fractions of similar K_{av}, whose Stokes' radii had been established earlier. Apparently the radius of gyration of the chondroitin sulphate molecules (mol. wt. 19,200) was approximately 25% larger at very low ionic strength than at $I = 0.2$. It was concluded from these experiments that 0.2 M NaCl provides an adequate ionic strength for the chromatography of chondroitin sulphate; the effect of further increased salt concentration on the Stokes' radius was almost negligible. Therefore, the experiments described below were all carried out with 0.2 M NaCl as eluant, since at higher concentrations of salt the automated analysis of uronic acid was hampered by the formation of HCl gas.

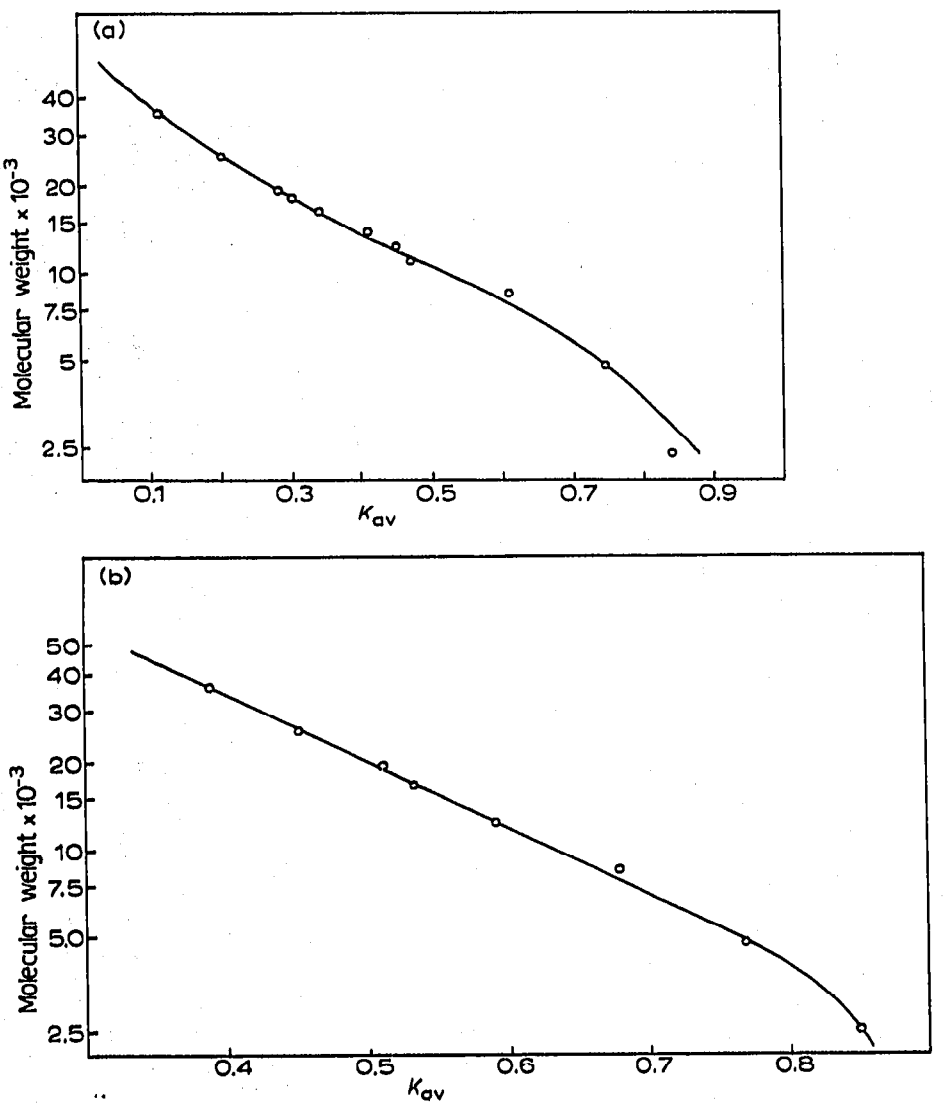

Fig. 1. Calibration of columns of Sephadex G-200 (a) and Sepharose 6B (b) with reference fractions of chondroitin sulphate. The molecular weights of the different fractions have been plotted *vs.* the respective K_{av} values.

Effect of polysaccharide concentration. Chromatographies of chondroitin sulphate on Sephadex were also obtained at different concentrations of the polysaccharide. A moderate increase in the polysaccharide concentration did not significantly affect the elution patterns. However, if more than 3 mg of chondroitin sulphate were charged on the column, corresponding to an increase in the concentration by a factor of about 20, the elution of the polysaccharide was retarded and the resulting profile distorted, indicating marked intermolecular interactions[33,34]. However, at the low concentrations generally used, such interactions were insignificant.

Calibration of gel columns. The K_{av} values of the reference fractions of chondroitin sulphate on the gel columns were determined from V_0, V_t and the respective elution volumes for the fractions[29], and were then plotted *vs.* the logarithms of the molecular weights of the respective fractions (Table I, Fig. 1). As can be seen from Fig. 1, slightly curved plots were obtained.

Determination of the molecular weight and molecular-weight distribution of chondroitin sulphate

The established relationship between the molecular weight and the elution position (K_{av}) of chondroitin sulphate was used for determining the molecular weights of unknown samples of the polysaccharide. The following procedure has been applied to the chromatography data[8]. The elution profile for the preparation was divided into a large number of segments (for practical reasons *e.g.* 20), the K_{av} values of which correspond to certain molecular weights, M_i, according to the calibration curve (Fig. 1). The relative amount of chondroitin sulphate, W_i, within each fraction was calculated and used to derive the weight-average ($\bar{M}_w$) and number-average ($\bar{M}_n$) molecular weights of the whole preparation according to the equations:

$$\bar{M}_w = \Sigma W_i \cdot M_i \tag{1}$$

$$\bar{M}_n = \frac{1}{\sum \frac{W_i}{M_i}} \tag{2}$$

The cumulative molecular-weight distribution (Fig. 2) may be obtained by summing the amount of eluted material from the tail end of the chromatogram, *i.e.* from $K_{av} = 1$ to $K_{av} = 0$ and plotting this parameter *vs.* the molecular weight, obtained from K_{av} using the calibration curve (Fig. 1). By differentiating this function, the corresponding differential molecular-weight distribution is obtained (Fig. 2).

Resolution

The resolution of the Sephadex G-200 was studied by the following control experiments. A preparation of ^{35}S-labelled chondroitin sulphate ($\bar{M}_w = 18{,}300$; $\bar{M}_n = 14{,}800$) was fractionated on the column (Fig. 3). A subfraction was selected from a portion of the chromatogram, where zone-spreading would strongly interfere with the calculation of the molecular dispersion, and was then rechromatographed on the same column. The mol. wt. (13,200) of the selected molecular population, as determined from its elution profile (Fig. 3), was only slightly higher than that expected from its original elution position (12,500). This result indicates that the shape and

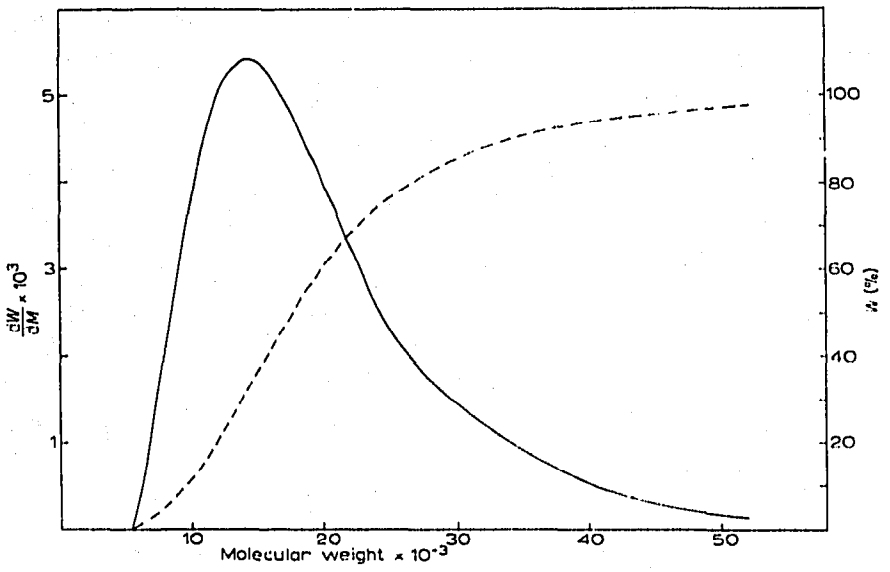

Fig. 2. The molecular-weight distribution of chondroitin sulphate from bovine nasal septum. The amount of eluted material has been accumulated from the tail end of the chromatogram (W) and plotted *vs.* molecular weight (- - - -). The total amount of material is taken as 100%. This curve has been differentiated to give the corresponding differential molecular-weight distribution function, dW/dM (———).

width of an eluted chondroitin sulphate peak essentially reflects the molecular dispersion of the preparation rather than a zone-broadening phenomenon. The validity of the method was also tested with two polydisperse chondroitin sulphate samples, composed of known amounts of well-characterized, practically mono-

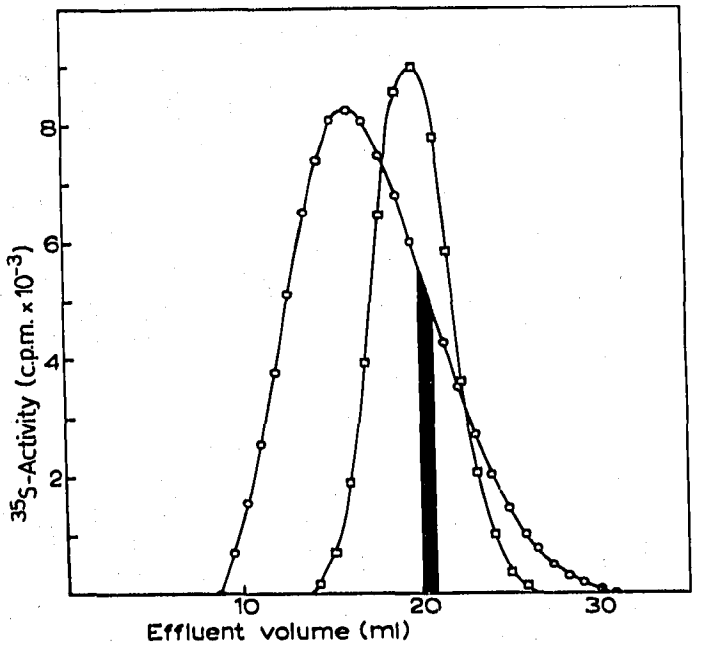

Fig. 3. Chromatography of ^{35}S-labelled chondroitin sulphate on a column of Sephadex G-200 (○—○). A subfraction corresponding to the shaded portion of the chromatogram was rechromatographed on the same column (□—□). The eluates were analysed for ^{35}S-activity. For comparison the activities of the rechromatographed sample were multiplied by a factor of 10.

disperse subfractions. One of these samples was a mixture, composed of equal amounts of fractions 2, 3, 4 and 5 (Table I). The $\bar{M}_w$ (19,800) and $\bar{M}_n$ (16,200) values of this sample were determined by gel chromatography on the Sephadex column. Although they were in fair agreement with those calculated from the molecular weights of the constituent fractions (Table I) according to eqns. 1 and 2 ($\bar{M}_w$ = 18,800; $\bar{M}_n$ = 17,800), the ratio $\bar{M}_w/\bar{M}_n$ was higher, which may be ascribed mainly to the zone spreading on the gel column. A hyaluronidase-treated material, identical with that used for the preparation of low-molecular weight fractions of chondroitin sulphate[21], was also examined. The molecular-weight values obtained by gel chromatography were $\bar{M}_w$ = 9,700 and $\bar{M}_n$ = 5,700, as compared to the theoretical values of 10,700 and 8,700, respectively. The $\bar{M}_w$ values were thus in fair agreement. The discrepancy between the $\bar{M}_n$ values remains unexplained, but may conceivably reflect some degree of molecular dispersity in the constituent fractions. However, an additional and probably more important factor is the error due to zone-spreading, which is most pronounced in the low-molecular-weight region, where the slope of the calibration curve is steep.

Reproducibility

The reproducibility of the analytical system was investigated by repeated chromatography of two preparations of chondroitin sulphate from chick-embryo epiphyseal cartilage and bovine nasal septum, respectively. The molecular weights ($\bar{M}_w$) of the two tested preparations, determined 17 and 10 times, were 27,700 and 19,800 and the corresponding standard deviations 0.8% and 0.7%, respectively.

For the average of duplicate determinations of the molecular weight ($\bar{M}_w$), a 95% confidence interval of $\bar{M}_w \pm 3\%$ is obtained[35].

Applications of the method

The molecular weights of a large number of chondroitin sulphate preparations have been determined by the present method. Most of this work, covering both structural and metabolic aspects of the chain length of chondroitin sulphate, will be presented elsewhere, and only a few applications will be mentioned here. The molecular weights of chondroitin sulphates from various sources are given in Table III.

Comparatively high molecular weights were noted for chondroitin sulphate prepared from compact bone of normal and rachitic puppies[36], in agreement with the findings of HJERTQUIST AND VEJLENS[37]. Since the material was eluted with or near the void volume of the Sephadex column, these preparations were analysed on the column of Sepharose 6B, which gives a superior resolution in the high-molecular-weight range to that obtained with Sephadex G-200. By comparison chondroitin sulphate isolated from epiphyseal cartilage of 14-day-old chick embryos and from bovine nasal septa showed considerably lower molecular weights (see also BUDDECKE *et al.*[17] and LUSCOMBE AND PHELPS[12]).

Using the present technique the molecular weight of chondroitin sulphate was also studied in relation to the macromolecular properties of the chondroitin-sulphate proteoglycan. Protein-polysaccharide, isolated from bovine nasal septa[26], was separated on a preparative column of Sepharose 2B into four fractions (I–IV) as indicated in Fig. 4. These fractions, the molecular weights of which were estimated to range from approximately 1×10^6 to approximately 10×10^6 (ref. 38) were then degraded

TABLE III

MOLECULAR WEIGHTS OF CHONDROITIN SULPHATE FROM DIFFERENT SOURCES, DETERMINED BY GEL CHROMATOGRAPHY

$\bar{M}_w$ = weight-average molecular weight; $\bar{M}_n$ = number-average molecular weight.

Origin of chondroitin sulphate	*Remarks*	$\bar{M}_w$	$\bar{M}_n$
Compact bone of puppies	Prepared from normal animals, aged 3 months	44,000	26,000
Compact bone of puppies	Prepared from rachitic animals, aged 3 months	42,000	26,000
Chick epiphyseal plate	Prepared from 14-day-old embryos	29,000	23,000
Bovine nasal septa	Prepared by direct proteolytic digestion of the tissue	19,800	15,500
Bovine nasal septa	Prepared from PPL I[a]	17,900	13,600
Bovine nasal septa	Prepared from PPL II[a]	18,200	14,000
Bovine nasal septa	Prepared from PPL III[a]	18,000	13,700
Bovine nasal septa	Prepared from PPL IV[a]	17,800	13,500
Bovine nasal septa	Hyaluronidase-digested[b]	9,700	5,700

[a] See text and Fig. 4 for explanation.
[b] See WASTESON[21].

by proteolysis to yield single chondroitin sulphate chains (see MATERIALS AND METHODS). As seen from Table III the molecular sizes of the chondroitin sulphate fractions thus obtained were identical, and, as expected, in fair agreement with that of chondroitin sulphate isolated from bovine nasal septa by direct digestion of the tissue. It was concluded that the considerable variation in size of the protein-polysaccharide preparation was not the effect of a varying chain length of the polysaccharide moiety. TSIGANOS AND MUIR[39] arrived at similar conclusions by applying a different technique to the protein-chondroitin sulphate of pig laryngeal cartilage.

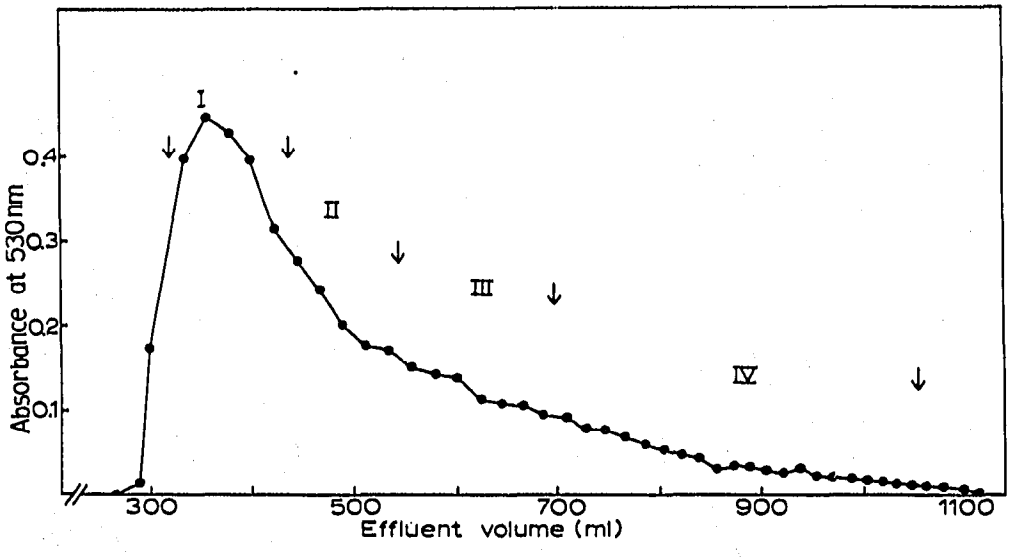

Fig. 4. Fractionation of PPL on a 4.2 × 84 cm column of Sepharose 2B. The effluent was analysed by the carbazole method and pooled into four fractions (PPL I–IV) as indicated by the arrows.

DISCUSSION

The present method for the determination of molecular weights by gel chromatography rests on the assumption that the elution position (K_{av}) of a compound is related to the molecular size. This relationship, which has been verified by several authors (for reviews see *e.g.* DETERMANN[2], JOHNSON AND PORTER[40], and LAURENT *et al.*[41]) may also be applied to the gel chromatography of chondroitin sulphate, as demonstrated by the calibration curves, shown in Fig. 1. Ideally, for a given gel, any K_{av} value should thus represent a certain molecular size. However, even minor variations in the experimental parameters may influence the elution behaviour, and, consequently, the experiments should be standardized as far as possible. Significant errors may thus arise from the use of too low an ionic strength (Table II) or from overloading of the column. The influence of temperature and flow rate has been discussed elsewhere[7,31,42].

The maintenance of proper experimental conditions is a prerequisite for the optimal performance of the gel column. However, even under optimal conditions chromatography of homogeneous molecular populations will yield peaks of finite widths. The significance of this zone-broadening phenomenon for the resolution of the analytical system has been discussed by several authors[40,43,44] and various corrective measures have been suggested[7,40,45,46]. The separation on a gel column due to difference in molecular size is proportional to the column length, whereas the zone spreading is proportional only to the square root of the column length. In consequence, the relative effects of zone spreading will decrease with increasing column length, as verified experimentally by WINZOR *et al.*[42,46]. In view of these considerations the column dimensions used in the present study would seem to be satisfactory. Nevertheless, an attempt was made to assess the effects of zone spreading in our system by rechromatographing a previously separated subfraction of chondroitin sulphate (Fig. 3). The resulting chromatogram showed that, for practical purposes, the effect of zone spreading could be neglected.

The resolving power of the analytical system was tested by chromatographing mixtures of well-characterized chondroitin sulphate subfractions (combined fractions 2–5 and 7–11, respectively). The results of these experiments demonstrated that the $\bar{M}_w$ and $\bar{M}_n$ values of polydisperse preparations of chondroitin sulphate may be adequately determined by the gel chromatography method. However, care should be taken in evaluating $\bar{M}_n$ values of chondroitin sulphate preparations eluted close to V_t, since the $\bar{M}_n$ value obtained for the hyaluronidase-treated preparation was probably too low. For samples of low dispersity, however, the reliability is probably sufficient for most biological problems, since the theoretical and experimental $\bar{M}_w$ values were in reasonable agreement.

Although the resolving power of the gel columns is optimal only within a limited fractionation range, the separation properties of Sephadex G-200 adequately cover the molecular-weight range of chondroitin sulphate[41]. While most chondroitin sulphates have molecular weights less than 30 000, some species, *e.g.* from bone, penetrate the upper limit of the separation range for Sephadex G-200. Therefore, for the high-molecular species of chondroitin sulphate, Sepharose 6B is preferred, since it is more permeable to large macromolecules than in Sephadex G-200. It should

be pointed out, however, that the overall resolution of Sepharose 6B is somewhat inferior to that of Sephadex G-200 (ref. 41).

The detailed characterization of chondroitin sulphate specimens, isolated from small amounts of tissue, is often limited by the supply of the polysaccharide. The small amounts of material required by the present method is one of the main advantages in relation to conventional methods for the determination of molecular weights, such as osmometry, light scattering or even ultracentrifugation. The relative simplicity and inexpensiveness of the analytical system further enhances the applicability of the present technique in relation to others. Finally, it should be noted that gel chromatography offers unique possibilities in metabolic studies for determining selectively the molecular weights of radioactively labelled polysaccharides.

The main drawback of the gel chromatographic method lies in the preparation and characterization of the reference substances required for the calibration curve. The necessity of using homologous preparations as references has been pointed out previously: the exclusion properties of a molecule on a gel column depend primarily on the size and form of the molecule and only indirectly on its molecular weight[41]. This is demonstrated by the relatively low K_{av} of chondroitin sulphate as compared to its molecular weight. Thus serum albumin (molecular weight 69 000) has the same K_{av} on Sephadex G-200 as a chondroitin sulphate with a molecular weight of 13,000. Further it should be borne in mind that variations in the properties of the gels and in the mode of operation may preclude the direct application of a calibration curve, obtained in a different laboratory.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Medical Research Council (B70-13X-4-06B), the Swedish Cancer Society (53-B69-04X), Konung Gustaf V:s 80-årsfond, Svenska Sällskapet för Medicinsk Forskning and the Medical Faculty of the University of Uppsala.

The skilful technical assistance of Miss B. Hiort and Miss C. Sellin is gratefully acknowledged.

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