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APPLICATION OF THE SORPTION PROPERTIES OF SPHERON GELS IN HIGH-RESOLUTION LIQUID COLUMN CHROMATOGRAPHY OF NATURALLY OCCURRING MACROMOLECULAR SPECIES

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SUMMARY

Sorption effects, in addition to molecular sieving properties, of Spheron P 1000 gel (grain size less than 25 μm) were exploited for the separation of complex naturally occurring mixtures of high-molecular-weight compounds. The applicability of this approach was demonstrated on the separation of glycosaminoglycans and proteoglycans. In both examples, separations exceeding those obtained with conventional sorbents and ion exchangers were achieved.

INTRODUCTION

Ethylene glycol methacrylate–ethylene glycol dimethacrylate copolymers prepared by Kubín *et al.*[1] and in the spherical form suitable for chromatography by Čoupek *et al.*[2] are among the few examples of semi-rigid gels capable of withstanding rather high pressures and therefore suitable for high-pressure and high-resolution techniques. In biochemistry, moreover, and in related fields their hydrophilic properties are extremely valuable, especially at present when high-resolution liquid column separations of hydrophilic substances are rather scanty, while just the reverse is true with hydrophobic compounds[3].

In preliminary tests with these gels, some sorption effects have been observed that were due to the partially hydrophobic character of the polymer matrix. With polysaccharides and polypeptides the separations occur purely on the molecular

sieving basis. The high exclusion limit of the Spheron 1000 gel offers the possibility of gel permeation separation of rather high-molecular-weight substances such as polysaccharides[2]. Until now the sorption properties of these gels were considered a disadvantage, because of the absence of a clear interpretation of results on the molecular-weight basis, and were neglected. However, in some instances, as demonstrated in the experimental part of this work, sorption effects can be exploited for excellent separations of yet unresolved or poorly resolved compounds.

Good examples of mixtures of hydrophilic, naturally occurring polymers that are hard to separate are protein–polysaccharide complexes and glycosaminoglycans (for a review, see ref. 4). None of the procedures so far proposed for the separation of glycosaminoglycans, such as the cetylpyridinium procedure[5], ECTEOLA-cellulose chromatography[6], chromatography on DEAE-Sephadex[7] or Dowex 1-X2 (ref. 8), offers a complete separation of all the basic types, *e.g.* chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate and keratan sulphate. The situation with the separation of protein polysaccharides from connective tissue and similar types of compounds is currently even worse. The only successful results in this field were obtained by isoelectric focusing[9,10]. Few attempts have yet been made to separate these categories of compounds by high-resolution techniques.

Therefore, a search for effective methods of separating naturally occurring mixtures, at least of the first two categories of compounds, seemed highly desirable with a concomitant attempt to elucidate the role of the proteinous and carbohydrate parts of the molecules in the separation process itself.

EXPERIMENTAL

Materials

Sorbent. Spheron 1000 was prepared in the Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia[11].

Compounds used for calibration. Hyaluronic acid and chondroitin sulphate (BDH, Poole, Great Britain); dextrans of definite average molecular weight (range 2.10^6–1.10^4) (Pharmacia, Uppsala, Sweden); fibrinogen (human) (Armour Labs., Great Britain); albumin (bovine) (Sigma, St. Louis, U.S.A.); alcohol dehydrogenase (EC 1.1.1.1) and cytochrome *c* from horse heart (Reanal, Budapest, Hungary); trypsin (EC 3.4.4.4) and soy-bean trypsin inhibitor (Koch-Light, Colnbrook, Great Britain).

Proteoglycan complexes. Cartilage proteoglycans were prepared as follows. Human (and also chicken) articular cartilage was homogenized in liquid nitrogen and the homogenate was extracted three times with a fivefold excess of 4 *M* guanidinium hydrochloride each time over a period of 24 h. The reaction mixture was centrifuged, the supernatant discarded and the resulting sediment was digested with pronase for 24 h (0.01 % solution in 0.1 *M* calcium acetate). The undissolved residue was freed from the supernatant fluid and extracted twice for 24 h by tenfold excess of 0.45 *M* sodium chloride. After each extraction, the supernatant was collected by centrifugation. Then the residue was similarly extracted with 0.8 *M* sodium chloride[12]. All the extracts were pooled, dialysed overnight against 0.05 *M* Tris–HCl buffer (pH 7.4, 0.02 *M* with respect to sodium chloride) under gentle stirring. The dialysed sample was then applied to a DEAE-cellulose column and the proteoglycan fraction

was isolated by the method described previously by Miller[13], by eluting the column with 0.05 *M* Tris–HCl buffer 1 *M* with respect to sodium chloride. Before this buffer was applied, the column was eluted with the same buffer that was used for dialysis in order to elute the contaminating collagenous fraction. Proteoglycans formed a single peak and no further resolution was obtained under these conditions.

Buffers. Sodium tetraborate and potassium dihydrogen phosphate used for the preparation of appropriate buffers were products of Lachema (Brno, Czechoslovakia).

Apparatus

Three stainless-steel columns, 125 × 0.8 cm I.D., packed with Spheron 1000 (grain size less than 25 μm) were used in series. The mobile phase was pumped by a piston pump MC-706 B-300 (Mikrotechna) which was capable of developing an over-pressure of 40 atp. For abolishing pulse disturbances the damping device from an Hd-1200 amino acid analyser was inserted into the feeding line. A six-way valve served as the sampling device with the 200-μl sample loop. Differential refractometer Knauer Model 2025 attached to eKN/T-1 VEB (Messgerät Erich Weinert) served for detection and recording. The columns were packed by the technique described previously by Heitz and Čoupek[14].

Methods

Aqueous dextran solutions (0.05%) were used for calibration with water as mobile phase. For calibration with proteins, 0.05 *M* sodium tetraborate–0.1 *M* potassium dihydrogen phosphate (pH 7.0) (buffer I) and 0.05 *M* sodium tetraborate–0.5 *M* potassium dihydrogen phosphate (pH 7.0) (buffer II) were used as mobile phase. Proteins were applied as 1% solutions in the above buffers. Proteoglycans were lyophilized before chromatography and redissolved in buffer I. To facilitate

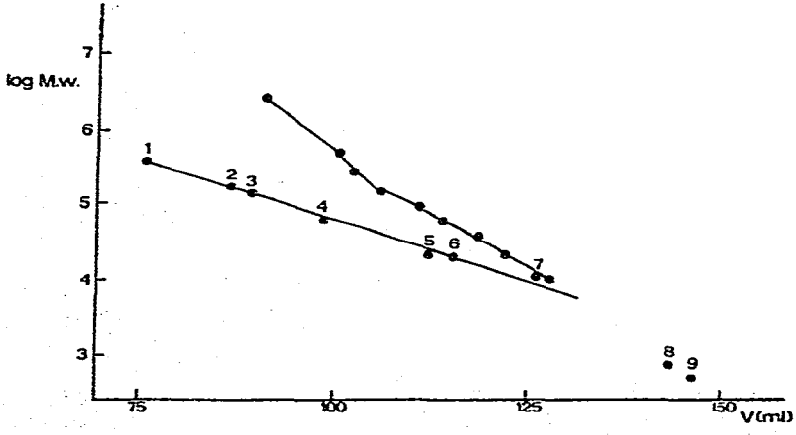

Fig. 1. Calibration of the system with dextran fractions of known average molecular weights 2 000 000, 500 000, 250 000, 150 000, 110 000, 70 000, 40 000, 20 000, 10 000, with a series of proteins and with sucrose and glucose. 1 = Fibrinogen; 2 = alcohol dehydrogenase; 3 = albumin, dimer; 4 = albumin, monomer; 5 = trypsin; 6 = soy-bean trypsin inhibitor; 7 = cytochrome *c*; 8 = sucrose; 9 = glucose. Mobile phase: distilled water for dextran fractions, sucrose and glucose, buffer II for proteins. Flow-rate 0.66 ml/min.

TABLE I

THE RELATION BETWEEN MOLECULAR WEIGHT AND RETENTION VOLUME AT DIFFERENT IONIC STRENGTHS OF THE MOBILE PHASE

Protein	*Molecular weight*	*Retention volume (ml)*	
		Buffer I	*Buffer II*
Fibrinogen	341 000	76.2	133.7
Serum albumin, dimer	134 000	89.3	114.1
Serum albumin, monomer	67 000	99.2	123.9
Trypsin	24 000	113.8	128.0
Cytochrome *c*	13 000	126.6	127.6

dissolving of some samples, a few drops of acetic acid were added to the sample, and the sample was heated to 60° for a few minutes. Buffer I served as mobile phase.

Except for the flow-rate *vs.* HETP relationship, the flow-rate was kept constant in all separations (0.66 ml/min). The measurement of the dependence of the number of theoretical plates on the flow-rate was done with a 1% solution of trypsin, cytochrome *c* and the first proteoglycan fraction in buffer I.

RESULTS

While calibration of the system with a series of dextrans with known average molecular weight results in a linear relationship, and similarly a reasonably linear

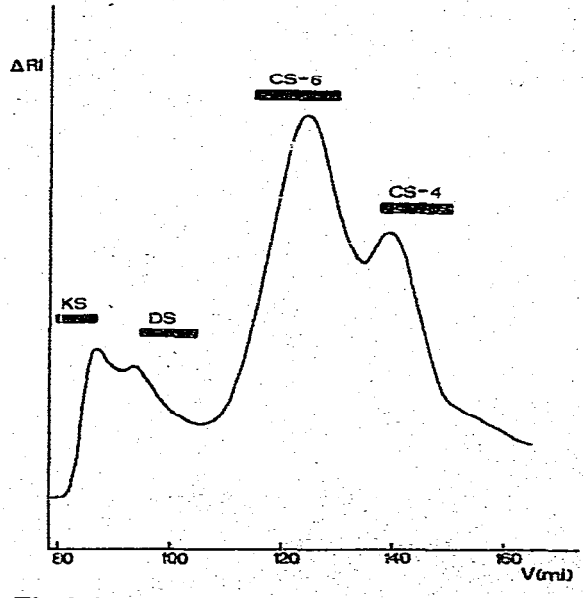

Fig. 2. Separation of isomeric chondroitin sulphates (CS-4 = chondroitin 4-sulphate; CS-6 = chondroitin 6-sulphate; DS = dermatan sulphate and KS = keratan sulphate on Spheron 1000. Mobile phase: buffer I; flow-rate 0.66 ml/min. For experimental details see *Methods*. Peaks were identified by appropriate standards and by analysing the types of carbohydrate represented in the individual peaks according to Antonopoulos *et al.*[5]. Fractions taken for analysis are designated by horizontal lines.

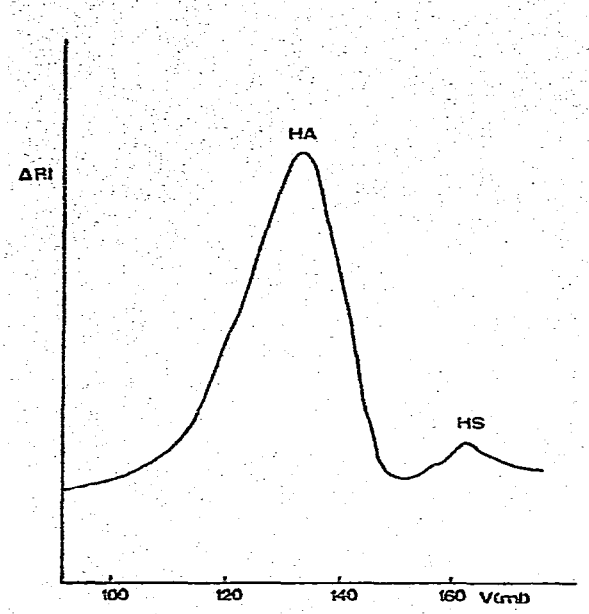

Fig. 3. Chromatogram of a hyaluronic acid (HA) sample on Spheron 1000 which proved to be contaminated with heparitin sulphate (HS). Mobile phase, buffer I; flow-rate, 0.66 ml/min. For experimental details see *Methods*.

relationship is obtained with proteins using buffer II (Fig. 1), at the lower salt concentration in the mobile phase (buffer I) distinct sorption effects occurred as indicated in Table I. When a mixture of glycosaminoglycans, mainly isomeric chondroitin sulphates, was applied to the system in buffer I, four distinct peaks were obtained with retention volumes 86.0, 92.6, 121.9 and 136.5 ml (Fig. 2). Based on these retention

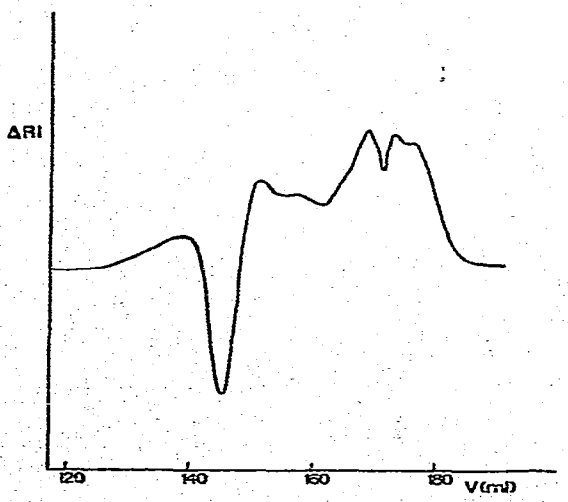

Fig. 4. Separation of human cartilage proteoglycans from a patient suffering from coxarthrosis. The forepeak with negative refraction index refers to acetic acid. Mobile phase, buffer I; flow-rate, 0.66 ml/min. For other details, see *Methods*.

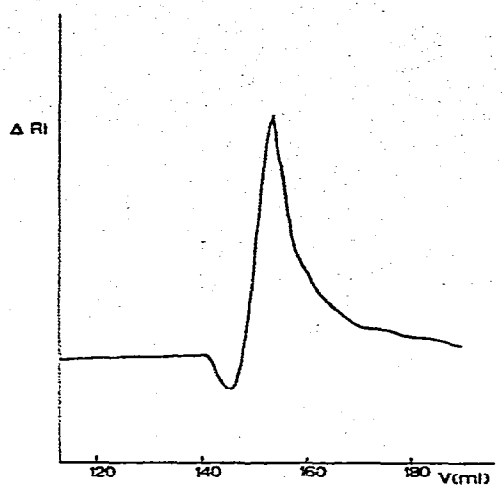

Fig. 5. Chromatographic profile of chicken proteoglycan fraction used for the HETP *vs.* flow-rate measurement, indicating the homogeneity of the fraction. The fore-peak with negative refraction index refers to acetic acid. Preparation procedure identical with that for human proteoglycans (see *Methods*). Mobile phase, buffer I; flow-rate, 0.66 ml/min.

data, the corresponding molecular weight estimates would be $> 2 \cdot 10^6$, $2 \cdot 10^6$, $2 \cdot 10^4$ and $4 \cdot 10^3$ if the separation had occurred on the molecular sieving basis only. (Since there were no differences in retention volumes of dextran fractions when water or buffer I was used as mobile phase, the calibration curve presented in Fig. 1 can be used.) However, when the salt concentration in the mobile phase was increased, no

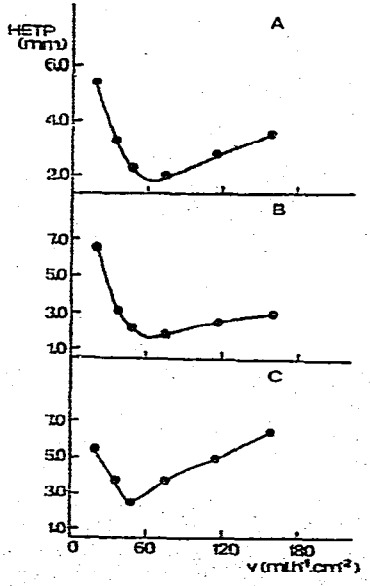

Fig. 6. HETP *vs.* flow-rate relationships for three different compounds (A = cytochrome *c*; B = trypsin; C = chicken cartilage proteoglycans on Spheron 1000. Mobile phase, buffer I.

separation of glycosaminoglycans occurred, indicating that other effects besides molecular sieving participate in separation. Besides, the above molecular weight estimates are in obvious disagreement with values obtained by other methods[15]. A similar separation of a sample of hyaluronic acid in buffer I did not result in discrete peaks, perhaps owing to the statistical polymerization of this macromolecule. In this separation sorption effects must also apply since the retention volume of 133.8 ml corresponds to a molecular weight of $9 \cdot 10^3$ (according to the dextran calibration) which is again in strong contradiction with molecular weight estimates obtained by other methods[16]. However, clear separation from contaminant heparitin sulphate was obtained (Fig. 3).

With increasing salt concentration in the mobile phase the quality of separation also decreased in the case of proteoglycans. In buffer I at least four distinct and well-reproducible peaks were obtained (Fig. 4), whereas when the concentration of potassium dihydrogen phosphate was increased to 0.5 *M*, a single broad peak was obtained.

The relation between the flow-rate and the number of theoretical plates for the two categories of substances separated, namely for trypsin and cytochrome *c* as pure proteins and for a chromatographically homogeneous proteoglycan fraction (Fig. 5), are summarized in Fig. 6, which indicates optimal flow-rates around 50 $ml \cdot h^{-1} \cdot cm^{-2}$ regardless of the chemical nature of the substances.

DISCUSSION

The present experiments were made to elucidate the question to what extent ethylene glycol methacrylate gels of the Spheron type are suitable for the separation of complex mixtures of hydrophilic biopolymers. Whereas with sugar polymers the dependence of the retention volume *vs.* molecular weight (in semilogarithmic plot) is linear, as reported earlier[2], and with proteins linearity of the same relation can be achieved at high salt concentrations in the mobile phase, at low concentrations distinct deviations occur, indicating the existence of strong sorption effects in proteins. Because high salt concentrations in the mobile phase cannot be generally applied with proteins, since some of them are easily susceptible to denaturation effects by high ionic strength, their behaviour at low ionic strength was investigated. The existence of the sorption effects in ethylene glycol methacrylate gels need not be a drawback, as this property can be exploited for the separation of complex mixtures in which high ionic strengths of the mobile phase are not desirable, as indicated for glycosaminoglycans and proteoglycans. The separations are superior to those reported up to now, and perhaps more detailed investigation on the nature of the mobile phase may bring further improvements in resolution.

HETP measurement in buffer I indicated excellent properties of the sorbent studied. The highest number of theoretical plates was achieved at a flow-rate of about 50 $ml \cdot h^{-1} \cdot cm^{-2}$, which corresponds to the equivalent height of about 2 mm. If one neglects the influence of grain size on the HETP, it is possible to compare the commercially available products as follows: for Sephadex G-200 (fine) the corresponding HETP value is 3.4 mm at 5.3 $ml \cdot h^{-1} \cdot cm^{-2}$, and with Ultrogel AcA 22 the corresponding value is 1 mm at 6 $ml \cdot h^{-1} \cdot cm^{-2}$ (ref. 17). The obvious advantage of the Spheron-type gels is the possibility of applying high flow-rates in comparison with

the two other types of gel. On the other hand, the V_t/V_0 value (V_t is volume of all available pores and V_0 is void volume) is rather low with Spheron 1000, requiring approximately ten to twenty times as many theoretical plates for equal quality of separation as with the other two types of gel. Therefore, fast separations can be performed at the expense of using high pressures and several columns combined in series. At the present level of instrumentation this is not a serious obstacle and rational combination of the sorption properties of Spheron gels with the sieving effects offer a wide variety of possibilities in separating complex mixtures of biopolymers. This statement was justified for proteoglycans, whose separation was incomparably better on Spheron than on Sepharose 4B or even on Sepharose 4B coupled to collagen[18].

The same is applicable for glycosaminoglycans, whose separation on Spheron gels exceeded that in methods using conventional sorbents and ion exchangers[5–8]. The possibility of rational combination of sorption effects with molecular sieving offers numerous possibilities of separation and purification of sulphated polysaccharides, proteins, glycoproteins and proteoglycans.

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