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OBSERVATIONS ON MOLECULAR WEIGHT DISCRIMINATION BY FILTRATION THROUGH POROUS MEDIA

J. F. KENNEDY
Department of Chemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT (Great Britain)

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SUMMARY

Investigation of the use of Bio-Gel P (cross-linked polyacrylamide) as the matrix for gel filtration of proteins and glycosaminoglycans with water as eluent revealed the operation of adsorption phenomena which could be overcome using an ionic medium as eluent. Similar investigations of porous glass indicated the operation of repulsion (negative adsorption) effects in water, and demonstrated the association of protein with the glass, even in the presence of an ionic medium. Possible causes of the adsorptions recognised are discussed and compared with those previously reported for adsorptions on to Sephadex (cross-linked dextran) gels.

INTRODUCTION

The use of supposedly inert porous matrices such as cross-linked dextran, agarose, cross-linked polyacrylamide, cross-linked polystyrene, porous silica and porous glass for the column fractionation of mixtures of molecules, according to their molecular size, in aqueous or organic media is a universally established procedure, the commercially available forms of these media being Sephadex, Bio-Gel A and Sepharose, Bio-Gel P, Bio-Beads S, Porasil and Bio-Glas, respectively. The technique essentially operates on the simple principles that smaller molecules of a mixture of molecular entities passing through a column of the matrix will penetrate the pores of the matrix more readily than larger molecules, and so will be retarded to a greater degree as elution of the column continues. Careful control of the pore sizes allows series of media to be produced which will fractionate over defined molecular weight ranges. However, it is known that the separations obtained are not entirely based on the absolute molecular weights of the compounds under investigation, but also depend upon the tertiary structure or overall shape and size of the molecule. The porous media recommended are supposedly inert, and therefore, theoretically, interactions with the molecules being fractionated should not occur.

However, many reports have now appeared indicating the interaction of molecules with Sephadex gels during fractionation experiments. Various investiga-

tions demonstrated unexpected retardation of certain classes of compounds on fractionation upon Sephadex gels, *e.g.* aromatic and heterocyclic compounds on G-25 using water as eluent[1], aromatic amino acids on two gels using buffers as eluents[2], phenylalanine peptides on G-10 in 0.5 *M* acetic acid[3], peptides containing aromatic amino acids on G-25 in water[4] and G-15 in 0.2 *M* acetic acid[5], and streptomycin on G-10 in water[6]. Thus it was apparent that under the conditions of investigation factors additional to the permeation of the molecule into the matrix were operating. Clearly the effect of these factors is to delay the elution of the compound from the column, and so to give the impression that its molecular weight is less than that in reality.

Furthermore, these abnormal phenomena associated with Sephadex gel chromatography were not confined to the more closely linked dextran gels. The initial fractionation of polydisperse human IgG on Sephadex G-150 in 0.004 *M* sodium acetate buffer pH 5.4 gave one peak only, the higher molecular weight material being adsorbed[7]. A second fractionation with the same column and an identical load gave two peaks, suggesting that certain active sites in the gel had been blocked in the first chromatogram.

These problems experienced with Sephadex gels and our observation that such gels tend to leak carbohydrate slowly prompted the investigation of the alternative matrices, Bio-Gel P and porous glass, for the particular work in hand—distinction between glycosaminoglycan samples resistant and susceptible to testicular hyaluronidase—for which molecular weight discrimination seemed appropriate. The observations and conclusions from the investigation are presented in this paper.

EXPERIMENTAL AND RESULTS

The following compounds were employed: dialysed human serum albumin (kindly provided by Dr. P. J. SOMERS), testicular hyaluronidase (Type II, salt free, Sigma Chemical Company), umbilical cord hyaluronic acid (Fluka Inc.) and hyaluronic acid hexasaccharide (kindly provided by Prof. S. A. BARKER), which had the following structure: β-D-GpA-(1 → 3)-β-D-GpNAc-(1 → 4)-β-D-GpA-(1 → 3)-β-D-GpNAc-(1 → 4)-β-D-GpA-(1 → 3)-D-GpNAc.

The following buffers were used: 0.01 *M* sodium phosphate buffer pH 7.30 in 0.2 *M* sodium chloride (buffer 1) and 0.09 *M* sodium barbiturate buffer pH 8.60 (buffer 2) prepared by addition of concentrated hydrochloric acid (approx. 1 ml/l) to sodium barbiturate solution and dilution to give the final pH and concentration stated.

Glass columns (45 × 1.3 cm diameter) of Bio-Gel P-30 (100–200 mesh) were prepared in the usual way, the dry gel being swollen in water for 24 h at 4° before being packed. Columns were eluted with the appropriate eluent prior to use to achieve equilibration of the gel. Loads (1000 μl) were applied using the usual layering technique. During fractionations, which were always carried out at 4°, columns were eluted at a flow rate of about 20 ml/h, and fractions (1 ml) were collected.

Glass columns (88 × 0.85 cm diameter) of porous glass (Type Z, 50–100 mesh, 1850 Å pore diameter, kindly provided by Dr. W. HALLER) were prepared by deaerating the beads under water *in vacuo* and keeping them submerged during the packing process, which was carried out at room temperature (20°). The bead bed was settled by the repeated tapping of the column until the bed height contracted no further. The porous glass was then cleaned before use by the passage of concentrated nitric acid

(5 ml) through the column followed by washing with distilled water until the eluate was of pH 5.5, and equilibrating with the appropriate eluent at 4°. Loads (1000 μl) were applied and fractionations carried out as for the Bio-Gel P column using a flow rate of about 30 ml/h. The cleaning procedure was repeated after use of the column.

The following load sizes were generally used: human serum albumin, 3.0 mg; testicular hyaluronidase, 2.0 mg; hyaluronic acid, 200 μg; hyaluronic acid hexasaccharide, 200 μg. Samples were dissolved in the system which was subsequently used for their elution from the columns, and appropriate duplicate aliquots for quantitative determinations were removed before application to the columns.

The techniques employed for monitoring the column fractions involved the determination of UV absorption at 280 nm for protein, and the carbazole assay[8] in a microscale form for hyaluronic acid and hyaluronic acid hexasaccharide. Quantitative assays of fractions, summation of the values under a peak, and comparison with the load applied permitted determination of the recoveries of materials from the column.

The results of individual fractionation of the various compounds on the columns using distilled water or the buffer systems described as eluents are summarised in Table I and Figs. 1–6.

DISCUSSION

The glycosaminoglycans can be broadly classified according to their susceptibility (hyaluronic acid, chondroitin, chondroitin 4-sulphate and chondroitin 6-sulphate) and resistance (dermatan sulphate, heparin, heparan sulphate and keratan sulphate) to testicular hyaluronidase.

The aim of the present investigation was to provide a simple method whereby the susceptibility of a glycosaminoglycuronan or mixtures of glycosaminoglycuronans to testicular hyaluronidase could be determined. The glycosaminoglycans in their protein-free forms generally have a molecular weight of 14,000 or greater, and the products from testicular hyaluronidase treatment of the susceptible glycosaminoglycans are mixtures of the di-, tetra- and hexasaccharides, the molecular weight of the non-sulphated and sulphated hexasaccharides being 1152 and 1392, respectively. It was therefore necessary to choose matrices whose operational fractionation ranges were approximately 2000–14,000. The molecular weight fractionation ranges of the matrices chosen appeared appropriate according to the information from the suppliers.

Achievement of a suitable separation using water as eluent for the fractionation was desirable, since such would avoid unnecessary contamination of the sample and possible interference in subsequent assays. However, it was evident (Fig. 1) that adsorption phenomena were operative when Bio-Gel P-30 was used. Whilst use of human serum albumin as a marker for void volume determination fulfilled this object, extensive tailing of the peak occurred. Furthermore, testicular hyaluronidase was found to be partially retarded. Although this was a commercial and somewhat heterogeneous preparation, previous observations indicated that it should have been eluted completely in the high molecular weight region. The unsuitable nature of the Bio-Gel P/water system was highlighted by the extensive association of hyaluronic acid with the gel (Fig. 1). The adsorption phenomena observed did not cause irreversible retention of the materials by the column. Recoveries of human serum albumin and hyaluronic acid were virtually complete (Table I), whilst the lower value for

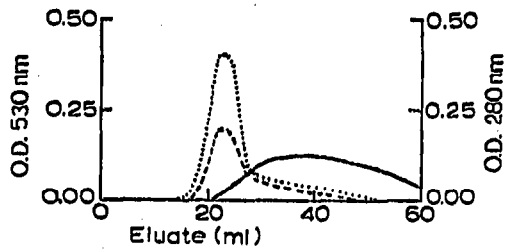

Fig. 1

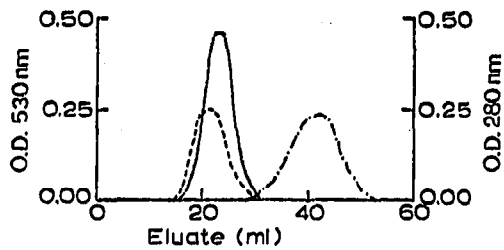

Fig. 2

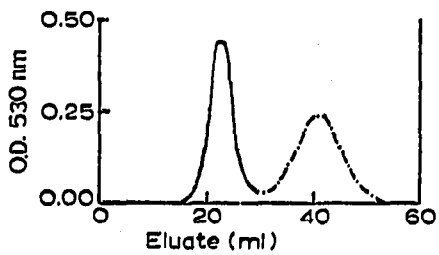

Fig. 3

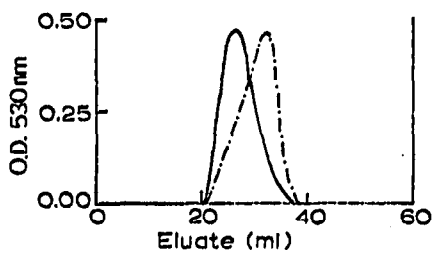

Fig. 4

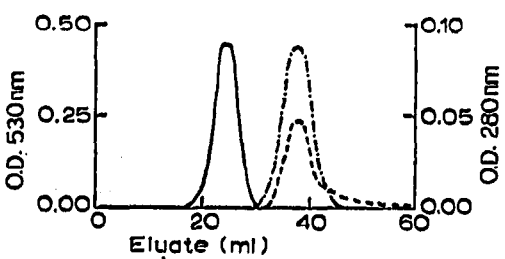

Fig. 5

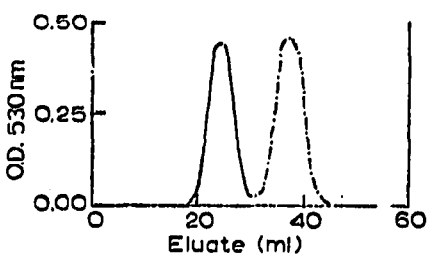

Fig. 6

Fig. 1. Fractionation of human serum albumin (·····), testicular hyaluronidase (— — —), and hyaluronic acid (———), on Bio-Gel P-30/water (separate elutions).

Fig. 2. Fractionation of testicular hyaluronidase (— — —), hyaluronic acid (———), and hyaluronic acid hexasaccharide (— · — · —) on Bio-Gel P-30/buffer 1 (separate elutions).

Fig. 3. Fractionation of hyaluronic acid (———) and hyaluronic acid hexasaccharide (— · — · —) on Bio-Gel P-30/buffer 1 (elution of mixture).

Fig. 4. Fractionation of hyaluronic acid (———) and hyaluronic acid hexasaccharide (— · — · —) on porous glass/water (separate elutions).

Fig. 5. Fractionation of testicular hyaluronidase (— — —), hyaluronic acid (———) and hyaluronic acid hexasaccharide (— · — · —) on porous glass/buffer 1 (separate elutions).

Fig. 6. Fractionation of hyaluronic acid (———) and hyaluronic acid hexasaccharide (— · — · —) on porous glass/buffer 1 (elution of mixture).

hyaluronidase may simply represent inaccuracies in integration over a number of peaks. The adsorption phenomena were clearly overcome in each case when an ionic medium was used for the fractionation (Fig. 2). Thus testicular hyaluronidase, hyaluronic acid and hyaluronic acid hexasaccharide were all separately and quantitatively eluted from the column in single discrete peaks. Fractionation of a mixture of hyaluronic acid and its hexasaccharide gave the elution profile expected from those obtained for the individual fractionations (Fig. 3).

From the results obtained for the use of porous glass, adsorption phenomena

TABLE I

RECOVERIES OF MATERIALS ON FRACTIONATION ON BIO-GEL P AND POROUS GLASS

Matrix	*Eluant*	*Material loaded*				
		Human serum albumin	*Testicular hyaluronidase*	*Hyaluronic acid*	*Hyaluronic acid hexasaccharide*	*Hyaluronic acid and hyaluronic acid hexasaccharide*
Bio-Gel P	Water	98%[a] (1)[b]	82% (1)	97% (1)	—	—
	Buffer 1	—	93% (2)	99% (2)	100% (2)	100% (3)
Porous glass	Water	—	—	94% (4)	102% (4)	—
	Buffer 1	—	17% (5)	97% (5)	97% (5)	97% (6)
	Buffer 2	—	48%	—	—	—

[a] Percentage recovery as determined from quantitative assay of aliquots of the column load and the fractions.
[b] Figure number.

were less immediately apparent when water was used as eluent, but a comparison of the elution profiles for hyaluronic acid and hyaluronic acid hexasaccharide shows an unexpectedly poor degree of resolution (Fig. 4). The recoveries of the carbohydrates were quantitative (Table I). Use of an ionic medium for fractionation markedly improved this resolution, and comparison of the new elutions indicated that forces other than diffusion had been operating during the water fractionation (Fig. 5). However, in spite of the fact that the recoveries of uronic acid containing materials were still quantitative, and that a good separation of these was achieved, a low recovery of enzyme protein was observed (Table I). Furthermore, the small proportion that was eluted from the column was eluted in the position of the hexasaccharide rather than the hyaluronic acid (Fig. 5). Use of a buffer of higher pH only moderately improved the protein recovery, the elution position being unaltered. Thus it would appear that in the use of porous glass, adsorption phenomena which operated in the case of water as eluant can be overcome by use of an ionic medium so far as acidic carbohydrate material is concerned (Fig. 6), but that forces still operate which bind protein.

As has already been indicated, various compounds exhibit affinity for dextran gels, and the adsorption phenomena have been classified[1] as: (A) those related to the structure of the substance and largely independent of the nature of the solvent, basic substances being adsorbed and acidic substances exhibiting repulsion (negative adsorption), and (B) superimposed effects dependent on the conditions of the column, *i.e.* ionic strength and pH of the aqueous phases, basic substances being adsorbed, and acidic substances being partly excluded from the matrix. Type "A" adsorption was considered to depend on charge involvement, there being a small number of carboxyl groups in Sephadex[9]. Type "B" adsorption was further demonstrated by the fact that at acid pH, basic amino acids were also excluded from the gel, in the absence of salt. In the case of dextran gels, it has been clearly demonstrated that the adsorptions are often reversible, thus peptides containing aromatic amino acids gave normal elution profiles when fractionated on Sephadex G-25 in phenol–acetic acid–water (1:1:1)[4].

These adsorption phenomena are not altogether undesirable, and have been put to good use. The first six members of a homologous series of phenylalanine peptides

have been separated by adsorption chromatography on Sephadex G-10 in acetic acid, the longer members being more highly retarded. Indeed, since ionic factors are involved, it was predicted that the ionised and neutral forms of weak electrolytes should have different rates of migration, and hence different elution volumes on beds of the tightly cross-linked dextrans according to the pH of the eluting solution, and this was utilised to determine the dissociation constants of some organic acids[10]. Adsorption effects have also been used for the separation of the geometrical isomers maleic and fumaric acids[11], and have been suggested as a means of increasing the separability of gel chromatography using Sephadex LH-20[12].

Explanations have been offered for the sources of affinity of compounds for dextran gels. The source of aromatic affinity to dextran gels was attributed to ether or hydroxy-ether groups in the cross-linking[13], whilst the adsorption of phenols, anilines and benzoic acids was attributed to the hydrogen bonding of the amino, carboxyl and hydroxyl groups to the hydroxyl group of the gel cross-linkages[14]. In contrast, evidence was presented for the adsorption of purines being due primarily to interaction with the dextran portion of Sephadex[15].

Bio-Gel P is claimed by the manufacturers to be inert, although since it is a cross-linked polyacrylamide, it could be considered very weakly acidic, on account of its amide groups. In the present work, it is quite obvious that the structure of the compounds partly determined the extent of adsorption to Bio-Gel P. The association of hyaluronic acid would appear to depend on ionic forces, since it has been shown that neutral oligosaccharides[16,17], including the chitin oligosaccharides[18], are not adsorbed by Bio-Gel P-2. Thus it may be concluded that neither hydroxyl, acetamido nor aldehydic (from reducing terminals) groups cause the association, but that the uronic acid carboxyl groups do. Furthermore, it is arguable that Bio-Gel P can be regarded as a pseudo-protein, and it is believed that hyaluronic acid associates with proteins. The groups causing the adsorption in the case of hyaluronidase are uncertain, particularly as albumin showed only a slight degree of association. However, the author has repeatedly experienced retardation of aromatic containing peptides and proteins on Bio-Gels (type P) on fractionation in water.

In the case of porous glass, a comparison of the elution positions of hyaluronic acid hexasaccharide fractionated in water and in buffer indicates the operation of repulsion (negative adsorption) forces in the case of water, whilst the hyaluronic acid positions in the two fractionations were virtually identical. This can be explained in terms of the hyaluronic acid being eluted in the void volume, but the overall operation of repulsion forces being due to the likely induction of acidic groups in the porous glass by the nitric acid treatment. Such acidic groups would repel the acidic carbohydrate but could easily cause adsorption of a protein via its ε-amino groups. Use of an ionic medium could be expected to overcome ionic reactions, but since only the carbohydrate was then recovered quantitatively, it would appear that the attractive forces were very strong, or that even covalent absorption could have occurred. The use of nitric acid for cleaning porous glass is recommended by manufacturers, but should, in the light of the foregoing, be used with caution. The effect of blocking any acidic groups with, *e.g.*, ammonia before fractionation was not investigated, although equilibration of the porous glass in a buffer of higher pH before fractionation of testicular hyaluronidase did not result in an increased recovery.

In conclusion, it may be stated that use of an ionic medium for the fractionation

of acidic carbohydrates is essential, and that Bio-Gel P is preferable to porous glass if proteinaceous material such as an enzyme is also present.

The Bio-Gel P-30/buffer 1 system has been successfully used on a microscale for the broad classification of acidic mucopolysaccharides according to their hyaluronidase susceptibility by bulking and concentrating the fractions corresponding to the polymer and oligomer elution regions before application of the carbazole assay. Results for chondroitin 4 and 6-sulphates, dermatan sulphate and heparin were according to those expected from the previously stated susceptibilities to the enzyme.

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