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IONIC EFFECTS IN THE CHROMATOGRAPHY OF PROTEINS ON COLUMNS OF CONTROLLED-PORE GLASS

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SUMMARY

Bovine serum albumin, acetylcholinesterase and two non-protein markers were chromatographed on columns of controlled-pore glasses in buffer solutions of varied ionic strengths. Adsorption of protein to the glass and ionic effects due to repulsion of the negatively-charged solutes from the glass matrix were observed. When glasses were treated with Carbowax 20M, the adsorption disappeared but ion exclusion effects persisted. The ionic behaviour of Carbowax-treated glasses was similar to that observed by one of the authors when using agarose columns.

INTRODUCTION

The controlled-pore glasses developed by Haller[1] have the advantages of mechanical strength and chemical stability which make them potentially superior to dextran, agarose or polystyrene gels for molecular exclusion chromatography. The main disadvantage encountered in their use is the strong adsorption of many proteins[2] and viruses[3] to the glass matrix, apparently by hydrogen bonding[3,4] of solute –NH or –OH groups to the oxygens of the –Si–O–Si– chains of the glass, and ionic bonding of solute cationic groups to the charged, non-bridging oxygen atoms of the glass network[2,4]. The first type of adsorption can be overcome by coating the glasses with polyethylene oxide[3] or polyethylene glycol[2], but ionic effects may remain[2]. Such ionic interactions are of great importance in the chromatography of polyelectrolytes such as proteins, but have not, as yet, been fully investigated. This paper is therefore concerned with a study of the exclusion of negatively charged solutes from the negatively charged pores of controlled-pore glasses both before and after treatment with polyethylene glycol[2]. The exclusion has been studied at various ionic strengths of the eluting buffer, and the results are compared with those previously obtained on agarose gels.[5]

EXPERIMENTAL

The column packings used were Corning controlled-pore glasses of actual

pore diameters 8.9, 13.9, 17.6, 23.5, 43.7 and 65.8 nm. The particle size was 120–200 mesh, and the pore volume was stated to be 0.54 to 1.37 ml/g for the various samples. Treatment with polyethylene glycol (Carbowax 20M; Union Carbide, New York, N.Y., U.S.A.) was carried out as described by others[2]. The buffer solutions used consisted of 5 m*M* Tris with NaCl as stated, adjusted to pH 8.0 with HCl at 20°. The column was of glass 2.12 cm^2 cross-sectional area and was packed to a height of approximately 23 cm. Extensive washing of the column with the buffer solution was required before use, and also when the ionic strength of the solution was changed. Thus when the buffer was changed from a mixture of NaCl and Tris to 5 m*M* Tris (both at pH 8.0) the pH of the effluent rose to pH 9.5 before slowly returning to the buffer pH of 8.0, an effect ascribed to the ion-exchange properties of the glass. Samples of a total volume of 0.5 ml were applied directly to the top of the packing which was then connected to a pump delivering buffer at 50 ml/h. Sample loads were 2.5 mg Blue Dextran 2000, 1 mg bovine serum albumin and 0.5 mg of a preparation of erythrocyte acetylcholinesterase (AChE; Sigma, St. Louis, Mo., U.S.A.). N-Dinitrophenylethanolamine (DNP-ethanolamine) was used as 0.2 ml of a saturated solution in buffer. Fractions of 2.0 ml were collected and analyzed as described previously[5]. All work was performed at 20°.

The partition coefficient (K_d) of the solutes into the pore volume of the matrix was determined from the formula

$$K_d = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the elution volume of the sample, V_0 the void volume measured as the elution position of the first excluded peak of Blue Dextran, and V_t is the total fluid volume of the column bed, estimated from the elution volume of DNP-ethanolamine.

RESULTS AND DISCUSSION

Chromatography on untreated glasses of varying pore size

Blue Dextran, DNP-ethanolamine and AChE were chromatographed on glasses of a range of pore sizes, from 8.9 to 65.8 nm, in buffers of two ionic strengths, *viz.* 0.1525 and 0.0025 (ref. 5). DNP-ethanolamine was always eluted as a sharp peak at the expected total aqueous volume of the column (Fig. 1). Its behaviour was consistent with that of a small solute showing no interaction with the glass structure. Blue Dextran on glasses of pore size 23.5 nm and below chromatographed as a sharp peak at the void volume at both ionic strengths, indicating complete exclusion from the pores. For glasses of pore diameter 65.8 and 43.7 nm a partially resolved second peak, due to the lower-molecular-weight species of the Blue Dextran (average molecular weight 2×10^6) was observed in addition to a sharp peak at the void volume when eluted with the higher-ionic-strength buffer (0.1525). At low ionic strength (0.0025) only the peak at the void volume was obtained. This behaviour on the larger-pore glasses (partial inclusion at $I = 0.1525$ but complete exclusion at $I = 0.0025$) is consistent with the previous observations on agarose gels[5]. At the higher ionic strength (0.1525), AChE (assayed as enzymic activity) was not eluted from any of the grades of glass, but broad bands of AChE activity were eluted from the columns in the buffer

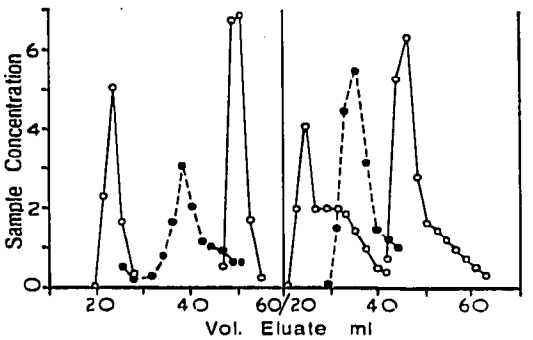

Fig. 1. Elution profiles obtained on 17.6-nm pore diameter glass at an ionic strength of 0.0025 (left) and on 43.7-nm glass at $I = 0.1525$ (right). The 43.7-nm glass was treated with Carbowax 20M. The peaks are, in order of elution, Blue Dextran 2000, AChE and DNP-ethanolamine. Ten units on the sample concentration scale have the following meaning for the various samples: Blue Dextran —one unit of extinction at 625 nm (left) or 0.5 units (right); AChE— a change of 0.25 (left) or 0.5 (right) units of extinction at 412 nm in 5 min when assayed by the thiocholine method; DNP-ethanolamine — one unit of extinction at 358 nm.

of $I = 0.0025$ (Fig. 1). Earlier bands were obtained from the smaller-pore glasses. These observations demonstrate the non-ionic adsorption described by others[3], which results in the loss of AChE at $I = 0.1525$, but also show ionic effects to be quite marked. Thus the elution of AChE at the lower ionic strength presumably results from the predominance of ion-exclusion effects[5–7] over non-ionic adsorption. The possibility that aggregation of AChE at low ionic strength had some effect under these conditions cannot be totally discounted, but was judged unlikely for reasons discussed previously[5].

Effect of varying ionic strength on chromatography

The elution of AChE and bovine serum albumin from a single grade of glass (13.9 nm) at a range of ionic strengths was then investigated. If molecular size only is considered, AChE would be expected to be eluted from this column near V_0, and albumin would be partially included in the pore space. At values of I between 0.001 and 0.0075, AChE when applied by itself was eluted as a broad band spread between V_0 and V_t. When applied to the column together with 1 mg of albumin, both proteins were eluted at V_0 in a sharp peak. When the ionic strength was 0.0525 the two proteins were eluted as broad peaks just after V_0. AChE exhibited a greater tendency to trail than did albumin. As noted previously, AChE was not eluted at $I = 0.1525$, and albumin was eluted as a broad band around V_t. These results are again explicable in terms of a non-ionic adsorption to the glass being opposed by ion exclusion at low ionic strengths. AChE seemed to be more strongly adsorbed than albumin, and the chromatography of the two proteins together resulted in blocking of the adsorption sites so that AChE elution was altered at low I.

Chromatography on Carbowax-treated glass

AChE was chromatographed on the 43.7-nm pore size glass after treatment with Carbowax 20M in a range of buffers of varying ionic strengths, together with Blue Dextran and DNP-ethanolamine (Fig. 1). There was no general adsorption to the

TABLE I

VARIATION OF K_d WITH IONIC STRENGTH ON A COLUMN OF CARBOWAX-TREATED 43.7-nm GLASS

Ionic strength (I)	*Partition coefficient (K_d)*
0.1525	0.50
0.1525	0.52
0.0225	0.42
0.0125	0.335
0.0075	0.25
0.0025	0.13

glass[2], and AChE was eluted at all ionic strengths. It was noticed that the peaks had a tendency to trail, the effect being most marked for DNP-ethanolamine, but also apparent for AChE and Blue Dextran. A sharp leading edge and initial rapid fall on the trailing side was followed by a long slow fall to the base-line, as shown on the right of Fig. 1. This indicated some degree of interaction of the samples with the Carbowax. Ionic effects seemed to control the elution position, as shown in Table I. Thus as the ionic strength of the eluting buffer was lowered, the AChE was progressively excluded from the internal pore volume[7] as demonstrated by the fall in partition coefficient K_d. These results fit the equation

$$K_d = \frac{k_1}{1 + \frac{k_2}{I}}$$

previously used by Crone[5] for the exclusion of AChE from Sepharose. A plot of $1/K_d$ against $1/I$ gave a good straight-line fit, from which values of $k_1 = 0.54$ and $k_2 = 0.0079$ were calculated. Ionic effects also influenced the elution of the Blue Dextran, for the partially included peak disappeared as the ionic strength was lowered.

The samples were also chromatographed on the 17.6-nm pore size glass treated with Carbowax in buffers of $I = 0.1525$ and 0.0025. The same effects as on the 43.7-nm grade were noted, except for a smaller shift in the AChE peak with I, due to the smaller pore size ($k_1 \simeq 0.16$). Trailing of the peaks was also marked on this column.

Use of Tween 20 in the buffer phase

As an alternative to Carbowax, the use of a non-ionic detergent (Tween 20) in the mobile buffer phase was investigated. Blue Dextran, AChE and DNP-ethanolamine were chromatographed on a column of 13.9-nm glass in a buffer of 5 m*M* Tris, 150 m*M* NaCl, 0.2% w/v Tween 20, pH 8.0. Blue Dextran was eluted as a sharp peak at V_0, and AChE (which was not eluted in the absence of Tween) was eluted as a broad band trailing back past V_t. DNP-ethanolamine was strongly adsorbed to the column and was eluted at $K_d > 10$. Tween 20 was thus analogous to Carbowax in causing a retention of some solutes in the stationary phase.

CONCLUSIONS

The ionic character of controlled-pore glass resulted in the exclusion at low

ionic strength of proteins and other molecules bearing a net negative charge. The ionic effects persisted after treatment with Carbowax 20M[2]. The relationship between elution volume and ionic strength fitted the empirical equation previously found to describe the same phenomenon on agarose gels[5]. Carbowax-treated glass could be used for the chromatography of proteins at an ionic strength (> 0.2) sufficient to obliterate the effect of the charges on the glass surface, but the additional peak trailing effect due to the Carbowax would spoil the resolution of complex mixtures. The recently described[8] chromatography of proteins as complexes with sodium dodecyl sulphate presents a further possibility in the use of controlled-pore glass columns. Alternatively, the chemical nature of the glass surface can be altered by substitution[7,9]; the use of such deactivated glasses for protein chromatography has not yet been fully evaluated.

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