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RESIDUAL ANIONIC PROPERTIES OF A COVALENTLY SUBSTITUTED CONTROLLED-PORE GLASS, GLYCERYL-CPG

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SUMMARY

A commercial controlled-pore glass medium for exclusion chromatography which is substituted with glycerol to eliminate non-specific adsorption (Glyceryl-CPG) was examined. Experiments on the elution of acetylcholinesterase and ganglioside micelles at varied ionic strength and pH showed that a slight anionic character still persisted on the glass matrix. At ionic strengths above 0.1, this had no effect on the elution of proteins. The material was found to have no tendency to adsorb proteins and other compounds, and was judged an excellent medium for exclusion chromatography. As a support for affinity chromatography, Glyceryl-CPG could be activated by periodate to form a virtually neutral matrix-ligand system.

INTRODUCTION

Various methods have been used to reduce the adsorption of proteins and other compounds to controlled-pore glasses, in an endeavour to increase the usefulness of these materials as media for molecular exclusion chromatography. The glasses have been coated with polyethylene oxide[1] or polyethylene glycol[2], have had the surface altered by chemical substitution[3], or have been used in conjunction with solutes such as sodium dodecyl sulphate (SDS)[4] or urea with SDS[5], which discourage adsorption to the glass. The use of glass with a chemically bonded coating is the preferable alternative, especially when it is wished to recover native protein from the column. Recently a controlled-pore glass substituted with glycerol (Glyceryl-CPG; Electro Nucleonics, Fairfield, N.J., U.S.A.) has become available. It was of interest to know whether ionic effects persisted on this material, as have been observed for Carbowax-treated CPG[6], and whether the material showed non-specific adsorption of protein[7,8] when used as a supporting matrix for affinity chromatography.

This paper describes the chromatographic behaviour of an anionic protein, acetylcholinesterase (AChE), and negatively-charged micelles of gangliosides on Glyceryl-CPG at different ionic strengths and hydrogen ion concentrations. This behaviour is compared with that of similar samples on unsubstituted CPG, reported

previously[6]. The use of Glyceryl-CPG for the affinity chromatography of AChE is also described.

EXPERIMENTAL

Two grades of glass, varying in their pore diameter, were used. Glyceryl-CPG of pore diameter 51.7 nm and particle size 120–200 mesh was used in a column of cross-sectional area 2.12 cm^2 and bed volume 48.5 ml. Buffer solution was pumped through at a rate of 34 ml/h at 20°. For experiments at varied ionic strength, the pH of the buffers was maintained at 7.4 with 5 or 10 m*M* Tris–HCl, and NaCl was added as required. To vary pH, the ionic strength was maintained at 0.0086, and the stated pH was obtained by systems of Tris–HCl, imidazole–HCl, 2-(N-morpholino)ethanesulphonic acid (MES) and sodium acetate–HCl. The samples were applied in 0.5 ml of the appropriate buffer and 1.7-ml fractions were collected. Sample loads were 2 mg of mixed gangliosides (Type III from bovine brain; Sigma, St. Louis, Mo., U.S.A.), 0.5 mg of AChE preparation (from bovine erythrocytes; Sigma), 1.6 mg Blue Dextran 2000 or 0.2 ml of a solution saturated with dinitrophenyl ethanolamine (DNP-ethanolamine). Glyceryl-CPG of pore diameter 17.7 nm was used in a column of cross-sectional area 0.64 cm^2 and bed volume 38 ml. It was eluted with a solution of 5 m*M* Tris, 150 m*M* NaCl at pH 7.4, flowing at 35 ml/h and 1.1-ml fractions were collected for analysis. Further experimental details are given in a previous paper[6].

An attempt was made to couple tri[^{14}C]-methyl-(*p*-aminophenyl) ammonium chloride hydrochloride[9] to the 51.7-nm pore diameter Glyceryl-CPG by the cyanogen bromide procedure as described by the manufacturer[10]. No coupling to the glass was achieved, despite minimal time delay between commencing to wash off cyanogen bromide and the addition of ligand (10 min). Because of this failure, activation by periodate was tried. Washed Glyceryl-CPG (pore diameter 17.7 nm; 1.0 g) was oxidized with 4 mmol sodium periodate. The activated glass was coupled with 40 ml 1 *M* 3,3′-diaminodipropylamine pH 5.0 and then reduced with 10 mmol sodium borohydride as described for Sepharose[11]. The oxidation and coupling were performed under nitrogen. A positive colour reaction with trinitrobenzenesulphonic acid[11] showed that the amine had been coupled to the glass. The glass (0.8 g) was then treated with 32 mmol succinic anhydride, and finally with 0.7 mmol trimethyl (*p*-aminophenyl) ammonium chloride hydrochloride and 2.2 mmol 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide methyl *p*-toluene sulphonate as described for Sepharose[12,13]. For affinity chromatography, this substituted glass was packed in a column of cross-sectional area 0.79 cm^2 to a bed volume of 2.2 ml. Crude Triton X-100 solubilized ox brain AChE[9] (1.2 ml) was applied to the column and eluted with buffer solution as described later. Fractions (1.8 ml) were assayed for AChE activity[14] and total protein[15]. The crude AChE preparation contained 3.15 mg protein per ml, and had a specific activity of 0.6 μmol acetylthiocholine hydrolysed per min per mg of protein (pH 8, 25°).

RESULTS AND DISCUSSION

The elution volumes of AChE and ganglioside micelles from the 51.7 nm Glyceryl-CPG column were dependent on the ionic strength of the eluting buffer at

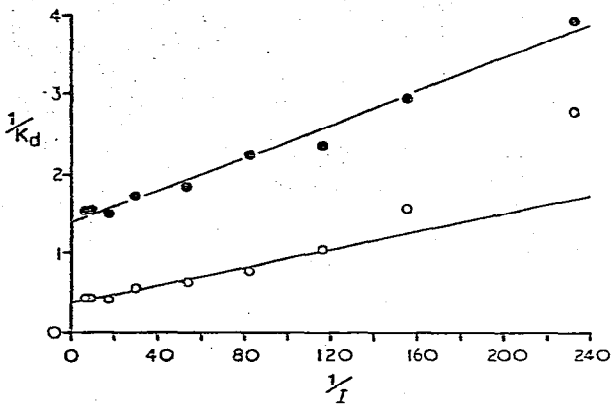

Fig. 1. Plot of $1/K_d$ against $1/I$ for (●) erythrocyte AChE and (○) mixed ganglioside micelles. The vertical scale should be multiplied by 4 in the ganglioside plot. The lines were calculated by the method of least squares. The two ganglioside results at low I were ignored when the line was calculated.

pH 7.4. The relationship between ionic strength (I) and distribution coefficient (K_d) was hyperbolic, as found previously for agarose[16] and CPG coated with Carbowax[6]. This relationship can be expressed as

$$\frac{1}{K_d} = \frac{1}{K_1} + \frac{K_2}{K_1} \cdot \frac{1}{I}$$

where K_1 and K_2 are constants. The plots of $1/K_d$ versus $1/I$ are shown in Fig. 1. The deviation from linearity observed for gangliosides at $I < 0.009$ is probably due to errors in determining K_d when V_e was near V_0. Estimates of K_1 and K_2 from this graph were 0.82 and 0.0074 for AChE and 0.79 and 0.0158 for ganglioside micelles (excluding the two results at low ionic strength). Slightly different values were obtained from analysis of a direct linear plot[17]; $K_1 = 0.78$, $K_2 = 0.0069$ for AChE, 0.75 and 0.0140 for gangliosides.

For reasons previously discussed[6,16] the variation of K_d with I is thought to be an ion-exclusion effect, by which the anionic protein AChE and the negatively-charged micelles of gangliosides are excluded from a negatively-charged matrix at low I. This argument is strengthened by the observation that a cationic protein, cytochrome c (pI 10.65), was eluted from the Glyceryl-CPG column as a sharp peak in buffer of $I = 0.154$ (77% recovery, $K_d = 0.99$) whereas it adsorbed to the top of the column at $I = 0.004$ and did not move further. Both experiments were performed at pH 7.4. These results lead to the conclusion that despite the covalent bonding of glycerol to the glass, some negative sites remain on the matrix.

The variation of pH at a fixed ionic strength (0.0086) led to results in agreement with this theory. The AChE was recovered from the column in good yield at pH values above 7 (Table I), but below 7 the yield was much lower than 100% and varied with the buffer used. Human erythrocyte AChE is reported[18] to exist in various forms with isoelectric points between pH 4.55 and 5.19 and that of the horse[19] is isoelectric at 4.65–4.7. By contrast the ganglioside micelles (pK of sialic acid groups[20] about 4) were eluted in good yield over the pH range stated, with no change in elution position.

TABLE I

THE EFFECT OF ELUENT pH ON THE CHROMATOGRAPHY OF ACETYLCHOLINESTERASE AND GANGLIOSIDES ON A COLUMN OF GLYCERYL-CPG 51.7 nm AT CONSTANT IONIC STRENGTH (0.0086)

n.d. = not determined.

Buffer system	*pH*	*Acetylcholinesterase*		*Gangliosides**
		K_d	*Recovery (%)*	K_d
Tris–HCl	8.35	0.45	100	0.24
Tris–HCl	7.4	0.475	100	0.27
Imidazole–HCl	6.95	0.475	102	n.d.
MES	6.15	0.39	72	n.d.
Imidazole–HCl	6.15	—	0	n.d.
Sodium acetate–HCl	5.5	0.53	16	n.d.
Sodium acetate–HCl	5.05	n.d.	n.d.	0.29
Sodium acetate–HCl	4.0	—	0	0.29

* Recovery of the gangliosides was between 80 and 100%.

The column matrix must bear a negative charge which persisted at pH 4 and bound or repelled the samples according to their net charge. Various samples were chromatographed on a column of Glyceryl-CPG (pore diameter 17.7 nm) in a buffer of pH 7.4 and ionic strength 0.154. The elution profiles are satisfactory (Fig. 2), and do not show the tailing evident on elution from Carbowax-treated CPG[6].

Methanol, glycerol and polyethyleneglycol 200 were all eluted at the same volume from the 51.7 nm Glyceryl-CPG column, and this corresponded to a total

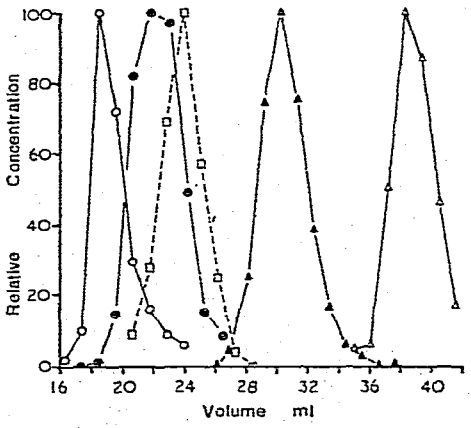

Fig. 2. Composite diagram of the elution of a number of compounds from a column of Glyceryl-CPG, average pore diameter 17.7 nm, in a solution of 5 m*M* Tris, 150 m*M* NaCl, pH 7.4 ($I = 0.154$) as described in the text. The concentration scales have been normalized so that the most concentrated fraction of each sample is 100 units. The samples, the load applied and the value of 100 concentration units are: ○, Blue Dextran 2000, 2 mg, $E_{1\,cm}$ (625 nm) 0.54; ●, erythrocyte AChE, 0.5 mg crude preparation, a change of 0.77 units of extinction at 412 nm in 5 min by the thiocholine method[14]; □, bovine serum albumin, 1 mg, 128 μg/ml; ▲, cytochrome *c*, 2 mg, $E_{1\,cm}$ (525 nm) 0.25; △, DNP-ethanolamine, 0.1 ml saturated solution, $E_{1\,cm}$ (358 nm) 0.27.

pore volume (V_t–V_0) of 19.0 ml, consistent with the value of 18.8 ml calculated from the manufacturer's data. These alcohols are therefore reliable markers of V_t, and it was noted that such hydroxylic compounds had no tendency to adsorb on to the bound glycerol of the matrix. On the other hand DNP-ethanolamine (initially used as a convenient marker of V_t because of its absorption at 358 nm) and benzyl alcohol had K_d values of 1.13 and 1.06, respectively, presumably due to a slight tendency of aromatic groups to adsorb on to the matrix.

The failure to couple a ligand to Glyceryl-CPG after cyanogen bromide activation was surprising. Successful activation by this reagent has been achieved by others[21] which may indicate that the technique we used was not adequate. The vicinal hydroxyl groups of the Glyceryl-CPG were successfully activated by sodium periodate[11]. The ligand was extended with succinic anhydride and coupled to trimethyl (*p*-aminophenyl) ammonium chloride hydrochloride as the affinity site. The chromatography of the solubilized ox brain AChE (summarized in Table II) was qualitatively similar to that observed for a similar sample on substituted agarose[9]. However, protein impurities were retained to a greater degree on the glass compared to the agarose, so that the specific activity of the AChE eluted with 1 *M* NaCl was only four times that of the starting material compared with a purification of at least 13-fold on agarose[9].

TABLE II

THE RECOVERY OF ENZYMIC ACTIVITY DURING THE AFFINITY CHROMATOGRAPHY OF OX BRAIN ACETYLCHOLINESTERASE (AChE)

All solutions contained 5 m*M* Tris and 0.05% Triton X-100 at pH 8.

NaCl concentration in eluent (mM)	*Volume of eluent (ml)*	*AChE activity (% of applied)*
0	10	14
100	10	8.5
200	10	9
1000	24	48
1000*	18	0.5

* With 5 m*M* edrophonium, a competitive inhibitor of AChE.

This work shows that the covalent bonding of glycerol to the glass does not eliminate entirely the negative character of the matrix which persists at least down to pH 4. In practice, this will not affect the chromatography of samples in buffers of ionic strength greater than 0.1, and the material represents a great advance over plain glass media which have high adsorptive properties and also over glass with a non-covalent coating. In fact, the ionic character can be utilized in the ion exclusion chromatography[22] of compounds, and is being used in this laboratory to study the interaction of gangliosides with proteins. As a support for affinity chromatography, Glyceryl-CPG has the advantage that an organic group is already attached to the glass, eliminating one step in the coupling of the ligand to the matrix. Activation by periodate is readily achieved, and is preferable to use of the toxic cyanogen bromide, which forms an unstable intermediate and introduces a charge in the product. The Glyceryl-CPG activated by periodate forms a virtually neutral affinity support.

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