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ION-EXCLUSION EFFECTS ON THE CHROMATOGRAPHY OF ACETYLCHOLINESTERASE AND OTHER PROTEINS ON AGAROSE COLUMNS AT LOW IONIC STRENGTH

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SUMMARY

Acetylcholinesterase (AChE), serum albumin and other proteins were chromatographed on columns of Sepharose 4B at varied ionic strengths of the eluting buffers, pH 8.4 (5°). AChE and serum albumin were progressively excluded from the gel as the ionic strength was lowered, whereas neutral or basic proteins showed no response or were adsorbed to the column. For the two acidic proteins (AChE and albumin) a hyperbolic relationship between K_{av} and ionic strength was found, and two empirical constants were calculated to define the relationship. It was concluded that the exclusion effects were due to the repulsion between the anionic proteins and anionic groups on the gel at low salt concentration.

INTRODUCTION

The unexpectedly early elution of acetylcholinesterase (AChE; EC 3.1.1.7) from agarose columns (Sepharose 6B) at low ionic strength was previously ascribed by the present author[1] to aggregation of the enzyme preparation under these conditions. Other workers[2] reached a similar conclusion when they chromatographed sialomucopolysaccharides on dextran columns (Sephadex G-200), and related effects were observed during the chromatography of inositol polyphosphates[3]. However, the latter authors attributed the early elution at low ionic strength to an anion-exclusion effect. In view of these conflicting conclusions, it was decided to study more thoroughly the chromatography of AChE and other proteins at varied ionic strength on agarose columns. The more porous agarose preparation Sepharose 4B was examined instead of Sepharose 6B used previously[1], as a wider range of elution volumes could be obtained at different ionic strengths.

EXPERIMENTAL

Proteins used and the source of supply were: Bovine serum albumin, bovine haemoglobin, chymotrypsin and cytochrome *c* from Nutritional Biochemicals (Cleveland, Ohio, U.S.A.); the soluble preparation of AChE from bovine erythrocytes (stabilized with buffer salts and gelatin) from Sigma (St. Louis, Mo., U.S.A.); electric

eel AChE from Worthington Biochemical (Freehold, N.J., U.S.A.); Triton X-100 from Rohm & Haas (Philadelphia, Pa., U.S.A.); the beaded agarose gel Sepharose 4B (Lot No. 9013) from Pharmacia (Uppsala, Sweden).

Two chromatographic columns were employed, with cross-sectional areas of 4.0 cm^2 and 5.3 cm^2. Each was fitted with top and bottom adaptors giving minimal dead space, and was packed to a total bed volume between 180 and 200 ml. The columns were packed with Sepharose 4B at room temperature in 150 m*M* NaCl, 5 m*M* Tris, 0.2% (w/v) Triton X-100 pH 8.0, then transferred to a cold room at 5°. The columns were conditioned by the passage of at least 2 l of buffer before being used for experimental observations. Samples of proteins (250 μg total protein of the AChE preparation and 7.5 mg albumin) in 1 ml of the appropriate buffer were applied, and eluted at a pump rate of 10 ml/h at 5°. Fractions of 5 ml were collected and analysed for AChE activity by the radiometric method[1], and for protein by the method of Lowry *et al.*[4] using bovine serum albumin as a standard. To avoid interaction between solutes, samples were always chromatographed individually. The eluant buffer was changed between runs by the passage of three column volumes of the new buffer before application of the sample. The buffer system principally employed consisted of 5 m*M* Tris, 0.2% (w/v) Triton X-100 with the desired concentrations of NaCl, adjusted finally to pH 8.0 at 20° with HCl (pH at 5° was then 8.4). For calculation of ionic strength, the contribution of Tris–HCl (p*K* 8.0 at 20°) was taken to be 0.0025. A sodium acetate buffer at pH 4.7 was also used. For this, 10 m*M* sodium acetate with NaCl was titrated to pH 4.7 with acetic acid. The ionic strength (*I*) was taken as 0.010 plus the NaCl contribution. Chromatography in acetate buffer was carried out at 20°. The volume of eluant was plotted against protein or AChE concentration in each fraction collected, and the elution volume (V_e) was determined as the centre of the peak at one third maximum height[5], a procedure adopted to minimize the random chopping action of the fractionator. Duplicate estimations of V_e were usually reproducible within 2 ml. The partition coefficient K_{av} was calculated from the formula $K_{av}=(V_e-V_0)/(V_t-V_0)$ where V_0 was the void volume and V_t the total bed volume of fluid and gel combined.

RESULTS

The relationship between elution volume and ionic strength

The results of the chromatography of AChE and other samples in buffer at different *I* are illustrated in Fig. 1. For erythrocyte AChE, a series of values of K_{av} were obtained at various values of *I* from 0.0025 to 0.1525, the variation being achieved by addition of NaCl to the 5 m*M* Tris buffer at pH 8.4. As found previously[1], K_{av} increased with increase in *I*, and on plotting the results as K_{av} *versus* *I*, a hyperbola was obtained, with rapid rise in K_{av} at low *I*, approaching a limiting value as *I* passed 0.10. From the equation for the hyperbola, it could be shown that

$$K_{av} = \frac{k_1}{1 + \frac{k_2}{I}}$$

Values of k_1 and k_2 were then obtained from the graph of $1/K_{av}$ *versus* $1/I$, in a manner similar to the treatment of the Michaelis equation for enzyme kinetics. The

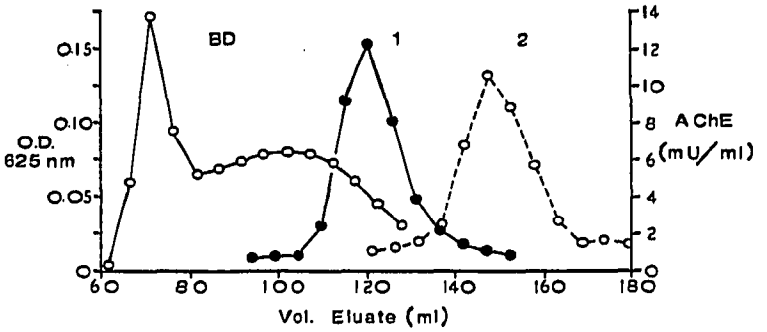

Fig. 1. Composite diagram illustrating the chromatography of Blue Dextran 2000 and AChE on a column of Sepharose 4B. Three separate chromatographic runs are illustrated. BD = Blue Dextran (1 ml of 0.5% w/v) showing a broad partially excluded peak with a sharp peak of totally excluded material at the void volume. $I=0.0055$. The left-hand scale refers to the optical density of the Dextran fractions. Peak 1 = Erythrocyte AChE at $I = 0.0055$. Peak 2 = AChE at $I = 0.0775$. The right-hand scale refers to AChE activity. The cross-sectional area of the column was 5.3 cm^2, with a total bed volume of 185 ml.

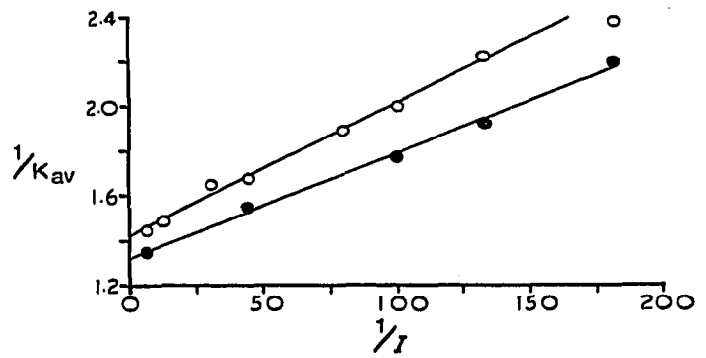

Fig. 2. Plot of the reciprocal of K_{av} against the reciprocal of ionic strength for the elution of erythrocyte AChE (open symbols) and bovine serum albumin (solid symbols) from Sepharose 4B. The lines were calculated by the method of least squares; the point for AChE at lowest I was ignored in the calculation.

graph for erythrocyte AChE is shown in Fig. 2. A good fit was obtained except for the two points at the lowest I values (one of which, at $1/I=400$, is not shown on the figure). At low ionic strength, small changes in pH and inaccuracies in concentration would produce proportionally greater variation in I, which may explain this deviation. The best line of fit to the points (ignoring the latter two) was calculated by the method of least squares, and from this values of $k_1=0.70$ and $k_2=0.0041$ were obtained. By analogy with the Michaelis equation, it can be seen that k_1 is the limiting value of K_{av}, and k_2 is the ionic strength which gives half that value. For electric eel AChE, values of $k_1=0.62$ and $k_2=0.0049$ were determined. Bovine serum albumin behaved in a manner very similar to AChE (Fig. 2), and values were obtained for $k_1=0.76$ and $k_2=0.0036$.

Blue Dextran 2000 was employed as a marker of the void volume (Fig. 1) but it became apparent that the elution of this substance was also influenced by the ionic

strength. The void volume (V_0) did not vary appreciably with I (Table I), but the distribution of the compound at V_0 decreased with increase in I, with the consequent augmentation of the partially included peak representing the mean value of the molecular weight distribution (Fig. 1 and Table I). Furthermore, the elution volume of this partially included peak increased with I, so that values of $k_1 = 0.45$ and $k_2 = 0.0054$ (seven observations at five values of I) could be obtained in the same way as for AChE.

TABLE I

EFFECT OF SALT CONCENTRATION ON THE ELUTION OF BLUE DEXTRAN 2000 FROM SEPHAROSE 4B

A sample of 1 ml of 0.33% (w/v) Blue Dextran 2000 was applied to a column of cross-sectional area 4.0 cm^2, height 54.5 cm, and eluted with 5 mM Tris–HCl, 0.2% (w/v) Triton X-100 with NaCl as indicated. V_e is the elution volume of the centre of the broad band of partially included Blue Dextran. Extinction at V_0 is the optical extinction at 625 nm of the Blue Dextran peak at the void volume.

NaCl concentration (*mM*)	V_0 (*ml*)	V_e (*ml*)	*Extinction at* V_0
0	73.9	92.5	0.338
5	71.6	110.5	0.245
5	71.9	110	0.212
150	72.4	136	0.108

The substances tested so far are all acidic: pI of bovine serum albumin = 4.4–4.8 (ref. 6); AChE from human red cells was reported to have pI below 6.0 (ref. 7), and electric eel AChE pI near 5.1 (ref. 8). Blue Dextran has three sulphonic acid groups in the chromophore[9]. Thus at pH 8.4, all these substances would be anionic. The effect of ionic strength of the buffer on the elution of neutral and basic macromolecules was therefore examined. Micelles of the non-ionic detergent Triton X-100 showed no dependence of K_{av} on ionic strength, neither did three neutral or basic proteins (Table II). Cytochrome *c*, the most basic substance, was strongly adsorbed to the column at low I, but moderate recovery was obtained at high I. Chymotrypsin was adsorbed to some extent at low I, and eluted fully at the higher value. The results of Table II are in accord with the general notion that the more basic proteins are adsorbed to the column at low I. In no case was a marked decrease in K_{av} observed at low I, comparable to that shown by albumin. The conclusion is that macromolecules with a net negative charge show a hyperbolic relationship between I and K_{av} whereas electrically neutral or positively charged molecules exhibit normal steric exclusion or adsorption to the agarose.

Effect of ionic strength at low pH

When chromatographed on Sepharose 4B at pH 4.7 in sodium acetate buffer, Blue Dextran was strongly adsorbed at all values of I tested (to 0.160). Very slow movement of a blue band down the column was observed, indicating a K_{av} well in excess of 2.0. Under the same conditions bovine serum albumin shows adsorption to the column at low I, with a return to normal chromatography at $I = 0.160$ (Table III).

TABLE II

ELUTION OF SOME PROTEINS AND TRITON X-100 MICELLES FROM SEPHAROSE 4B COLUMNS AT TWO IONIC STRENGTHS

Samples of the proteins (7.5 mg) or Triton X-100 (1 ml of 1% w/v solution) were applied to the columns and eluted with the Tris/NaCl buffer system described in Experimental at pH 8.4 (5°). The buffer used for protein elution contained 0.2% (w/v) Triton X-100; the buffer used for elution of the surfactant micelles contained 0.04% (w/v) of the surfactant to maintain micellar structure. Protein was estimated by method of Lowry *et al.*[4] and Triton X-100 by the extinction at 277 nm. The percentage of applied material which was recovered at the quoted K_{av} value is given. The figures for isoelectric point (p*I*) from ref. 6 refer to the values at $I=0.01$ and $I=0.10$–0.15 in that order at 25°.

Sample	*pI*	*I = 0.0075*		*I = 0.1525*	
		K_{av}	*Recovery (%)*	K_{av}	*Recovery (%)*
Bovine serum albumin	4.8–4.4	0.52	104	0.74	94
Haemoglobin	7.0–6.4	0.73	41	0.78	65
Chymotrypsin	8.6–8.1	0.83–1.11 *	69	0.92	100
Cytochrome *c*	?–10.65	∞ **	0	0.87	62
Triton X-100	–	0.68	86	0.71	84

* Eluted as a very broad zone with two maxima at these values.
** The cytochrome *c* was adsorbed to the top of the column.

TABLE III

ELUTION OF BOVINE SERUM ALBUMIN FROM A SEPHAROSE 4B COLUMN AT DIFFERENT IONIC STRENGTHS IN AN ACETATE BUFFER pH 4.7

Ionic strength	K_{av}	*Recovery (%)*
0.020	–	0
0.050	0.90	58
0.085	0.805	80
0.160	0.765	91

The K_{av} value (0.765) at this ionic strength compares well with the value at pH 8.4 under similar conditons (0.74). Albumin at pH 4.7 is electrically neutral (pI=4.8–4.4, ref. 6), and it displayed behaviour similar to that of the proteins neutral at pH 8.4 (Table II). The chromophore of Blue Dextran bears a variety of amino groups, the ionization of which may account for its adsorption to Sepharose at this pH.

Variation of the sample load on the columns

No significant change in K_{av} was observed when the amount of erythrocyte AChE (40 μg–4 mg total protein) or serum albumin (1–20 mg) applied to the columns was varied. Values of K_{av} for AChE of 0.45–0.465, albumin 0.50–0.52 and for electric eel AChE (0.35 μg protein) of 0.35 were determined. The experiments were performed in the pH 8.4 buffer at $I=0.0075$, so that K_{av} was at an intermediate value sensitive to changes in I.

Effect of change in the buffer salts on the elution of AChE

The NaCl normally used in the eluant buffer was replaced by a variety of inorganic salts (Table IV) and K_{av} measured for AChE under these conditions. The K_{av} was determined solely by the ionic strength; change in the anions or cations did not alter this value. Replacement of the Tris buffer with imidazole at pH 8.0 or 7.0 did not alter K_{av}, although recovery of AChE activity tended to be lower at the lower pH. Triton X-100 was originally included in the buffers for comparative work with the solubilized brain AChE, which required the presence of this detergent to maintain solubility[10]. However, omission of the Triton X-100 did not alter the results obtained. Table V demonstrates that the presence of Triton X-100 does not alter K_{av} values under identical conditions of ionic strength, and that K_{av} varies with I independently of the presence or absence of detergent.

TABLE IV

COMPARISON OF THE EFFECTS OF DIFFERENT INORGANIC IONS ON THE ELUTION OF AChE FROM A SEPHAROSE 4B COLUMN

The buffer used for elution consisted of 5 m*M* Tris + 0.2% (w/v) Triton X-100 with inorganic salts as shown adjusted to pH 8.0 (20°) with HCl.

Salt added to buffer	*I*	K_{av}
None	0.0025	0.32
10 m*M* NaCl	0.0125	0.53
2.5 m*M* $MgSO_4$	0.0125	0.555
30 m*M* NaCl	0.0325	0.605
30 m*M* KCl	0.0325	0.62
7.5 m*M* $MgSO_4$	0.0325	0.63
10 m*M* K_2SO_4	0.0325	0.615

TABLE V

ELUTION OF ERYTHROCYTE AChE FROM SEPHAROSE 4B IN THE PRESENCE AND ABSENCE OF TRITON X-100

The Sepharose column was equilibrated by the passage of an excess of the appropriate buffer, then the sample was applied and eluted at 5° as described in the text.

Buffer composition	K_{av}	
	0.2% (w/v) Triton X-100	*No Triton X-100*
5 m*M* Tris–HCl, pH 8.0 (20°)	0.32	0.29
5 m*M* Imidazole–HCl, pH 7.0 (20°)	0.29	0.31
5 m*M* Imidazole–HCl, 10 m*M* NaCl, pH 7.0 (20°)	0.54	0.505

Effect of ionic strength on the physical parameters of the column

As noted before, the void volume of the column did not change appreciably with the ionic strength of the buffer (Table I). Estimations of the total internal volume were performed by measuring the elution volume of the electrically neutral compound,

dinitrophenylethanolamine. This compound was eluted at $K_{av}=1.05$ and 1.04 in buffers of $I=0.0075$ and $I=0.1525$, respectively. After packing, the columns showed a continuous, very slight decrease in height over a period of weeks. Changes in ionic strength of the eluting buffer in either direction did not alter this trend. It was concluded that none of the physical parameters of the column (V_0, V_i or V_t) were significantly affected by changes in I capable of producing marked alteration in the elution of AChE.

Titration of acidic groups in the agarose gel

An attempt was made to titrate aliquots of the Sepharose gel in order to estimate the acidic groups present. Titration of 20 ml sedimented, washed gel suspended in an additional 10 ml of distilled water showed that there was a weak buffering power around pH 3.5–4.0. By titration between pH 2.5 and 7.0, the concentration of carboxylic groups was estimated very approximately as 2.6×10^{-6} equiv./ml of gel. Any sulphate groups present would not have been assayed by this method. It is stressed that the value of acidity estimated is extremely approximate, due to the technical difficulties of measuring it.

DISCUSSION

The agarose gel system consists of porous beads nearly completely penetrable by molecules the size of albumin, less so by AChE, but offering no steric barrier to inorganic ions. Pure agarose is electrically neutral, but residual contamination by agaropectin will be expected to contribute a low concentration of carboxyl and sulphate groups. There are a number of mechanisms by which such a system might show increased exclusion of macromolecules at low ionic strength, *e.g.* osmotic[11] and swelling effects due to sample solute or buffer medium, Donnan equilibrium effects originating from charged partially penetrant macromolecules, anion exclusion due to the acidic groups on the gel, and aggregation of the solute at low I. The first mechanism is unlikely, because the very small concentration of sample solutes used could not produce significant changes in the internal volume, and no gross change in V_i was produced by the changes in the buffer system. Furthermore, the same mechanism should then be operative for neutral macromolecules such as the Triton micelles, but in fact these were unaffected by alteration of I. A type of Donnan effect has been described by Fischer[12], which is independent of any fixed charges on the gel. Thus a partially excluded, charged macromolecule in the presence of penetrant counter-ions will set up a Donnan potential across the gel–liquid surface, and possibly influence the partition of the sample. However, it seems to the present author as if such a potential should tend to increase penetration of the macromolecule at low I, not exclude it as found in these experiments. Also, such a mechanism would be operative, with reversed sign, for positive as well as negative molecules, an effect not observed in this study. This reduces the possibilities to two: anion exclusion or aggregation as originally proposed by the author[1].

Aggregation seems unlikely for several reasons. The principal one is that no concentration dependence was found for a 20-fold variation in albumin sample sizes or 100-fold change in AChE samples, with a range in absolute protein weights from 350 ng (electric eel AChE) to 20 mg albumin. These studies were performed under

conditions in which variation in I caused marked changes in the elution pattern. Therefore it seems improbable that ionic strength influence the elution through an aggregation mechanism, which must necessarily be concentration dependent. Another argument against aggregation is that a continuous series of sharp peaks is observed, rather than a transition between a single, sharp peak of unaggregated material and a broad, polydisperse peak of aggregate. Further, the observed phenomenon can be linked to the ionic nature of the proteins, whereas there is no report in the literature of a continuous aggregation of albumin into larger and larger aggregates at reduced ionic strength. Some aggregation of AChE under these conditions may occur; Grafius and Millar[13] observed this phenomenon using AChE from *Electrophorus*, and Changeux[14] also noted aggregation of the enzyme from *Torpedo*. However, it would appear that in the present column technique, as opposed to the centrifugation technique used by others, different effects may be dominant and mask any aggregation.

The most reasonable conclusion is that the observed effects are caused by exclusion of the negatively charged macromolecules from the gel, due to the low density of negative charges bound to the agarose. On increase of ionic strength, the repulsion between the charges is reduced by the electrolyte to a point where the partition is determined solely by the steric factors ($I>0.2$). Such an effect would be independent of sample concentration and operative only for molecules negatively charged at the pH of the column, as observed. Positively charged molecules are bound to the gel at low I by electrostatic adsorption. Ion-exclusion effects on Sephadex have been reviewed by Janson[15], and the results of polynucleotide[16] and inositol polyphosphate[3] chromatography at varied I have been explained on this basis. It seems certain that the reported aggregation of sialomucopolysaccharides[2] in distilled water was in fact a result of ion exclusion. Sepharose gels would have a lesser charge density than Sephadex, but the author's previous observation of aggregation of AChE on Sepharose 6B was also probably an exclusion phenomenon. It can be calculated from the approximate figure for carboxyl group density on Sepharose 4B (2.6×10^{-6} equiv./ml) that this material has a charge density (excluding sulphate groups) less by a factor of 50 than an equivalent bed volume of CM-Sephadex C-50, a carboxylic cation exchanger. The negative charge on the agarose will presumably vary from batch to batch of gel, and also since some proteins seem to adsorb nearly irreversibly in small quantities to the gel, the behaviour of a particular column may vary according to the samples previously applied to it.

These results have general interest in that they emphasize the importance of maintaining the ionic strength of the buffer to at least 0.2. While the necessity of having salts present is generally recognized, it is apparent that some workers do not have a sufficiently high level. From the formula given under Results it can be shown that for a protein with $k_2=0.005$, an error of 1% in K_{av} will be present at $I=0.5$. As another example, Shafai and Cortner[17] report an apparent molecular weight of 4.2×10^5 for human erythrocyte AChE on Sepharose 4B in 0.05 M Tris, pH 7.2 ($I=0.043$). If the human AChE behaves in the same manner as the bovine enzyme, the molecular weight can be recalculated as 2.5×10^5 using the data from this paper. Theoretical interest in this work lies in the fact that it is possible to predict the behaviour of a solute on the column very precisely once two empirical constants have been determined. The first constant, k_1, is obviously a function of molecular size, whereas k_2 must reflect the electrical properties of the system. As far as AChE is

concerned, the report[1] of aggregation at pH 8.4 was unfounded; it seems that this column technique is not suitable for demonstrating such effects at low ionic strength.

REFERENCES

1 H. D. Crone, *J. Neurochem.*, 20 (1973) 225.
2 E. G. Brunngraber and G. Whitney, *J. Chromatogr.*, 32 (1968) 749.
3 J. H. Steward and M. E. Tate, *J. Chromatogr.*, 45 (1969) 400.
4 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
5 H. D. Crone, *J. Chromatogr.*, 60 (1971) 185.
6 R. A. Alberty, in H. Neurath and K. Bailey (Editors), *The Proteins*, Vol. 1, Part A, Academic Press, New York, 1953, p. 461.
7 C. A. Zittle, E. S. Dellamonica and J. H. Custer, *Arch. Biochem. Biophys.*, 48 (1954) 43.
8 W. Leuzinger A. C. Baker and E. Cauvin, *Proc. Nat. Acad. Sci. U.S.*, 59 (1968) 620.
9 *Blue Dextran 2000*, Pharmacia Fine Chemicals AB, Uppsala, Sweden, 1972.
10 H. D. Crone, *J. Neurochem.*, 18 (1971) 489.
11 E. Edmond, S. Farquhar, J. R. Dunstone and A. G. Ogston, *Biochem. J.*, 108 (1968) 755.
12 L. Fischer, in T. S. Work and E. Work (Editors), *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 1, North-Holland, Amsterdam, 1969, p. 348.
13 M. A. Grafius and D. B. Millar, *Biochemistry*, 6 (1967) 1034.
14 J.-P. Changeux, *Mol. Pharmacol.*, 2 (1966) 369.
15 J.-C. Janson, *J. Chromatogr.*, 28 (1967) 12.
16 Th. Hohn and H. Schaller, *Biochim. Biophys. Acta*, 138 (1967) 466.
17 T. Shafai and J. A. Cortner, *Biochim. Biophys. Acta*, 236 (1971) 612.